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RNAi studies reveal a conserved role for RXR in molting in the cockroach *Blattella germanica*

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Abstract

Ecdysteroids play a major role during developmental growth in insects. The more active form of these hormones, 20-hydroxyecdysone (20E), acts upon binding to its heterodimeric receptor, formed by the two nuclear receptors, EcR and RXR/USP. Functional characterization of USP has been exclusively conducted on the holometabolous insect *Drosophila melanogaster*. However, it has been impossible to extend such analysis to primitive-hemimetabolous insects since species of this group are not amenable to genetic analysis. The development of methodologies based on gene silencing using RNA interference (RNAi) after treatment with double-stranded RNA (dsRNA) in vivo has resolved such limitations. In this paper, we show that injection of dsRNA into the haemocel of nymphs and adults of the cockroach *Blattella germanica* can be used to silence gene function in vivo. In our initial attempt to test RNAi techniques, we halted the expression of the adult-specific *vitellogenin* gene. We then used the same technique to silence the expression of the *B. germanica RXR/USP* (*BgRXR*) gene in vivo during the last nymphal instar. BgRXR knockdown nymphs progressed through the instar correctly but they arrested development at the end of the stage and were unable to molt into adults. The results described herein suggest that RXR/USP function, in relation to molting, is conserved across the insect Class.

Keywords: Ultraspiracle/rxr; *Blattella germanica*; 20-hydroxyecdysone; RNAi; Molting

1. Introduction

In insects, developmental growth is periodically interrupted by molting cycles in which the animal enlarges its exoskeleton to accommodate internal growth. The molting process is primarily regulated by pulses of the ecdysteroidal hormone, 20-hydroxyecdysone (20E), upon binding to its heterodimeric receptor formed by two members of the nuclear receptor superfamily, EcR and the RXR-homologue ultraspiracle (USP). This functional heterodimeric receptor, in turn, elicits cascades of gene expression that mediate and amplify the ecdysteroidal signal.

Both EcR and USP have been characterized in great detail in holometabolous insects, especially in the dipteran *Drosophila melanogaster* (see Riddiford et al., 2001; King-Jones and Thummel, 2005). In this particular insect, the *EcR* gene encodes three protein isoforms, EcR-A, EcR-B1 and EcR-B2 (Talbot et al., 1993), whereas *USP* encodes a single protein (Oro et al., 1990; Henrich et al., 1990; Shea et al., 1990). Phenotypic analysis of three *USP* mutations has established the critical role of USP during early and late embryogenesis, as well as in larval molting and pupal development in *D. melanogaster* (Perrimon et al., 1985; Oro et al., 1992; Li and Bender, 2000; Hall and Thummel, 1998). Moreover, USP has been characterized in the dipteran *Aedes aegypti* (Kapitskaya et al., 1996; Wang et al., 2000) and in the lepidopterans *Manduca sexta* (Jindra et al., 1997) and *Bombyx mori* (Swevers and Iatrou, 2003).

Conversely, information regarding the functions of USP during the development of primitive-hemimetabolous insects remains very limited. Indeed, RXR/USP sequences have been cloned only in the orthopteran *Locusta*

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migratoria (Hayward et al., 1999, 2003) and in the dyctiopteran *Blattella germanica* (Maestro et al., 2005). Until now, functional characterization of these molecules has been hampered because these species are not amenable to genetic analysis. In recent years, however, the successful application of gene silencing by RNA interference (RNAi), following treatment with double-stranded RNA (dsRNA) in vivo, has led to unprecedented ways for dissecting complex gene functions on a molecular scale in non-model insects.

With the aim of elucidating the molecular basis of the role of 20E in nymphal development in hemimetabolous species, we previously cloned and characterized two cDNAs encoding RXR/USP homologues of the German cockroach, *B. germanica*, naming them BgRXR-S and BgRXR-L (Maestro et al., 2005). In the present work, we began the functional characterization of these factors in this cockroach. We first analyzed the developmental expression pattern of both receptors in tissues involved in molting, prothoracic glands and epidermis, and then developed a reliable RNAi in vivo approach. RNAi has been used in vivo in the cockroach *Periplaneta americana* to analyze the function of the homeotic gene *engrailed* in relation to the control of axon pathfinding and synaptic target choice in neurons of the cercal sensory system (Marie et al., 2000). To explore the possibility that RNAi in vivo was similarly effective in *B. germanica*, we tried to knock down the expression of the adult-specific *vitellogenin* (*BgVg*) gene. This gene was selected because it has been characterized in great detail in our laboratory during the last decade (Martín et al., 1995, 1998; Comas et al., 2000). Finally, the RNAi technique was used to silence the BgRXR in order to elucidate the functions of this protein in *B. germanica*. Our results showed that RXR plays a conserved role in controlling molting in this hemimetabolous insect.

2. Materials and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30±1 °C and 60–70% r.h. All dissections, injections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens.

2.2. Synthesis of dsRNAs and injection

To produce dsRNA targeting BgRXR (dsBgRXR) and BgVg (dsBgVg), PCR fragments for each transcript were cloned into pSTblue-1 vector (Novagen). For dsBgRXR, a 606 bp DNA encoding a protein sequence specific to the ligand-binding domain (LBD), from amino acid 217–413 (according to the sequence in the GenBank, accession number: AJ854489) was selected. This region within the LBD is common to BgRXR-S and BgRXR-L (see Fig. 3A), and hence it should knock down both transcripts at the same time. For dsBgVg, a 732 bp fragment from amino acid 746–990 (GenBank accession number: CAA06379) was used. As a control, a non-coding sequence of 92 bp from the pSTBlue-1 vector was used (dsControl). The respective RNAs were generated by in vitro transcription using either SP6 or T7 RNA polymerase from the corresponding plasmids and resuspended in water. To prepare the dsRNAs, equimolar amounts of sense and antisense RNAs were mixed, heated for 5 min at 90 °C, cooled down slowly to room temperature and stored at −20 °C until use. dsRNAs were suspended in diethyl pyrocarbonate-treated water and dissolved in Ringer saline with a final concentration of 1 μg/μl. Formation of dsRNAs was confirmed by running 1 μl of these reactions in a 1% agarose gel. A volume of 1 μl of the solution was injected into the abdomen of either newly emerged sixth instar nymph females (for dsBgRXR) or adult females (for dsBgVg).

2.3. Northern blot analyses of BgVg

To estimate BgVg mRNA levels, 5 μg of total RNA from adult female fat bodies were subjected to electrophoresis in 1.2% agarose gels containing formaldehyde, transferred to nylon membranes (Schleicher & Schuell), and hybridized, and then washed under stringent conditions. Hybridization was carried out with a specific probe for the *BgVg* gene that was labelled with fluorescein using the Gene Images random prime-labelling module (Amersham).

2.4. RT-PCR/southern blot analyses

RT-PCR followed by Southern blotting with specific probes was used to establish the expression pattern of BgRXR. Total RNA isolation from different tissues and cDNA synthesis were carried out as previously described (Cruz et al., 2003). PCR amplification of BgRXR was carried out in accordance with Maestro et al. (2005). Primers were as follows: forward (RXRF3), 5′-ATAATTGACAAGAGGCAGAGGAA-3′ and reverse (RXRR4), 5′-TGGTCACTAAGAGGCAAGGTAGT-3′. As a reference, the same cDNAs were subjected to RT-PCR/Southern blotting with a primer pair specific for *B. germanica* actin5C: forward, 5′-TCGTTCGTGACATCAAGGAGAAGCT-3′ and reverse, 5′-TGTCGGCAATTCCAGGGTACATGGT-3′, and with a primer pair specific for BgEcR-A (the heterodimeric partner of BgRXR; D.M., J.C., D.M-P. and X.B., unpublished results): forward, 5′-TACTCCGGAGGTAGCGTCATCAT-3′ and reverse, 5′-GACGGTGAAGACAACCAGTCATC-3′. cDNA probes for Southern blot analyses were generated by PCR with the same primer pairs using plasmid DNAs containing the corresponding cDNA clones as templates. The probes were labelled with fluorescein using the Gene Images random prime-labelling module (Amersham Biosciences). RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, indicating that there was no genomic contamination.

2.5. Microscopy and tissue analysis

All dissections of nymphal and adult tissues were carried out in Ringer's saline. To examine mouth parts, digestive tract and trachea, samples of these structures were directly immersed in glycerol 50% for microscopy. To study cuticle layers, the ventral cuticle was fixed in 4% paraformaldehyde (PFD), dehydrated, and paraffin embedded. Cuticle sections (6 μm) were stained with toluidine blue. Ovaries were fixed in 4% PFD, rinsed with PBS-0.2% tween (PBT), incubated for 10 min in 1 μg/ml DAPI in PBT, and mounted in mowiol. All samples were observed using a Zeiss Axiophot microscope, with images subsequently processed by Adobe Photoshop. Ovarian soluble proteins were quantified according to Bradford (1976).

3. Results

3.1. Silencing vitellogenin expression with RNAi

Activation of the *BgVg* gene in the fat body of adult females is cyclic, beginning 24 h after adult emergence and peaking around the middle of the reproductive cycle (Martín et al., 1998). Thus, for RNAi purposes, 1 μg of dsRNA targeting the BgVg sequence (dsBgVg) was injected into the abdomen of freshly ecdysed adult females; that is, just before the onset of *BgVg* gene activation. Specimens injected with dsControl were used as negative controls. Five days later, fat bodies were collected and mRNA levels of BgVg were estimated by Northern blot. BgVg mRNA was undetectable in dsBgVg-treated females whereas specimens treated with dsControl exhibited the levels expected of a 5-day-old adult female (Fig. 1A). We then analyzed the phenotypes of treated females by measuring protein levels in the haemolymph and the ovary (Fig. 1B and C). BgVg protein was undetectable in the haemolymph of dsBgVg-treated females, whereas it displayed normal levels in dsControl females. Protein content in basal oocytes of dsBgVg-treated females was reduced 77%, with respect to controls, which resulted in a dramatic reduction of oocyte length (Fig. 1D).

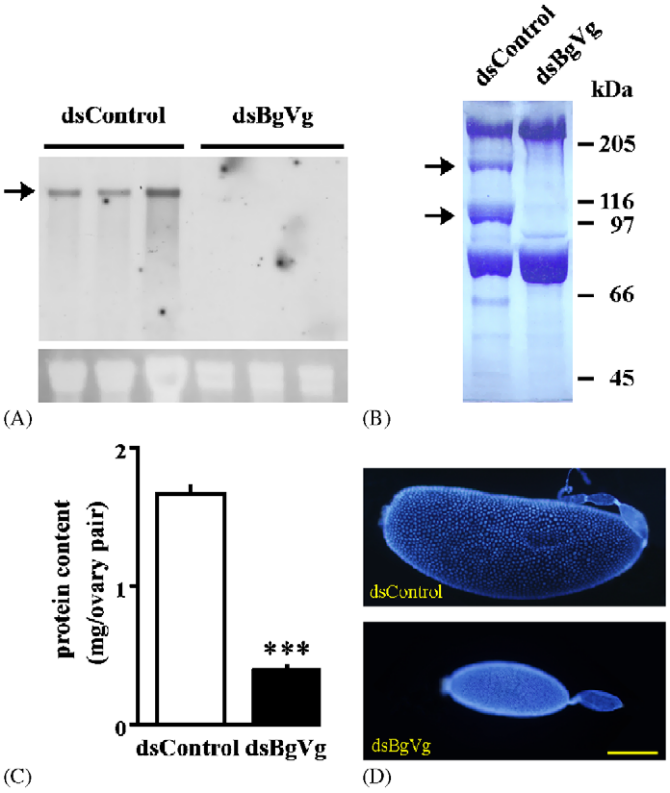

Fig. 1. Silencing *vitellogenin* (*BgVg*) gene expression by RNAi in vivo in adult females of *B. germanica*. Females were treated with dsBgVg or with dsControl just after the imaginal molt and were analyzed 5 days later. (A) Northern blot of total RNA (5 μg) extracted from fat bodies. Hybridization was carried out with a specific probe of the *BgVg* gene. Portions of the gel containing ribosomal RNA (rRNA) were stained with ethidium bromide to control for equivalent sample loading (lower panel). (B) SDS-PAGE of haemolymph. Molecular weight markers are indicated to the right, and subunits of BgVg are indicated to the left. (C) Total protein content in the ovaries. (D) DAPI staining of ovarioles. Scale bar: 500 μm.

3.2. Expression of BgRXRs during nymphal development

Once the experimental conditions for RNAi in vivo had been established, we applied this technique to analyze the functional role of BgRXRs during nymphal development in *B. germanica*. However, prior to RNAi experiments, and in order to determine the timing of dsRNA injection, we studied the developmental pattern of *BgRXR* gene expression in the prothoracic glands and in the epidermis, tissues involved in molting, during the fifth and sixth nymphal instars (Fig. 2). In both tissues, BgRXR-S was the predominant form. In fifth nymphal instar, the level of BgRXR-S mRNA declined during the mid instar in the prothoracic glands. Such decline was not observed in the epidermis. On the other hand, during the sixth nymphal instar, the expression of BgRXR-S in both tissues did not experience significant changes. The expression patterns of BgRXR-S and BgRXR-L contrasted with that of circulating

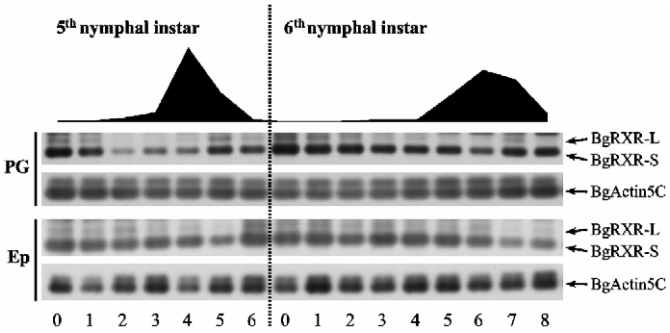

Fig. 2. Temporal expression patterns of BgRXR-L and BgRXR-S mRNA during the two last nymphal instars of *B. germanica* females. BgRXR mRNA levels were studied in prothoracic glands (PG) and in epidermis (Ep). Equal amounts of total RNA from staged nymphs were analyzed by RT-PCR/Southern blotting using BgRXR specific probes. BgActin5C levels were used as a reference. The blots shown are representative of three replicates. Above is represented the pattern of haemolymph ecdysteroids during these stages according to Cruz et al. (2003).

ecdysteroids, which exhibited a clear cyclic pattern during these nymphal instars (Fig. 2; data from Cruz et al., 2003).

3.3. Silencing RXR expression by RNAi

Taking into account the expression pattern of BgRXR, 1 μg of dsBgRXR (Fig. 3A) was injected into the abdomen of freshly ecdysed sixth instar female nymphs. As a negative control, we used female nymphs from the same group injected with dsControl. Prothoracic glands and epidermis with adhered fat body tissue of treated nymphs were collected, and mRNA levels of BgRXR were estimated by RT-PCR/Southern blot 7 days after treatment. Results (Fig. 3B) revealed that levels of both BgRXR mRNAs decreased in dsBgRXR-treated nymphs (87% reduction in prothoracic glands and 85% in epidermis/fat body) when compared with dsControl nymphs. Conversely, mRNA levels of BgEcR-A remained unchanged (Fig. 3B), thus providing an assessment of the RXR specificity for the silencing technique employed.

3.4. Phenotypic effects of RXR silencing

All dsBgRXR-treated sixth instar nymphs exhibited the same behaviour as dsControl specimens throughout the instar. However, at the end of the instar (day 8), 51% of dsBgRXR-treated specimens ($n = 41$) did not ecdyse and

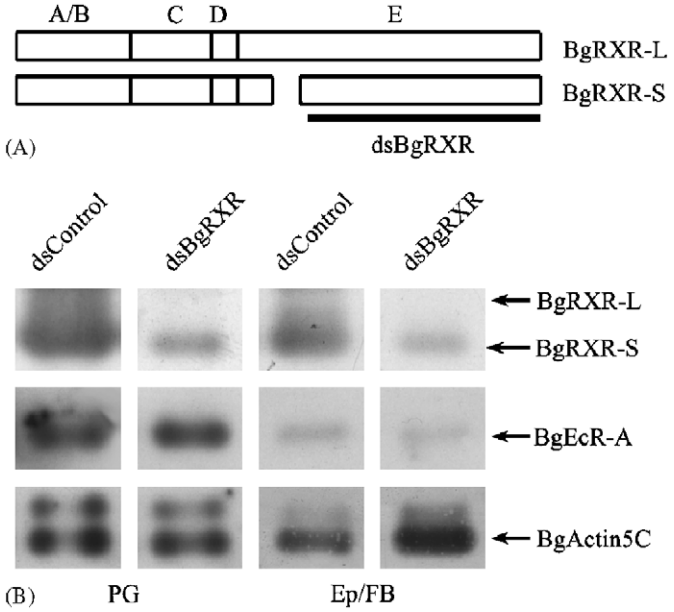

Fig. 3. Silencing *BgRXR* gene expression by RNAi in vivo in sixth instar nymph females of *B. germanica*. Freshly emerged sixth instar female nymphs were treated with dsBgRXR or with dsControl and were analyzed 7 days later. (A) Schematic representation of BgRXR-L and BgRXR-S showing the region that was used to generate dsRNA (dsBgRXR) within the LBD. (B) RXR mRNA levels in prothoracic glands (PG) and in the epidermis plus fat body (Ep/FB) studied by RT-PCR/Southern blotting. BgEcR-A and BgActin5C levels were used as a reference. The blots shown are representative of six replicates.

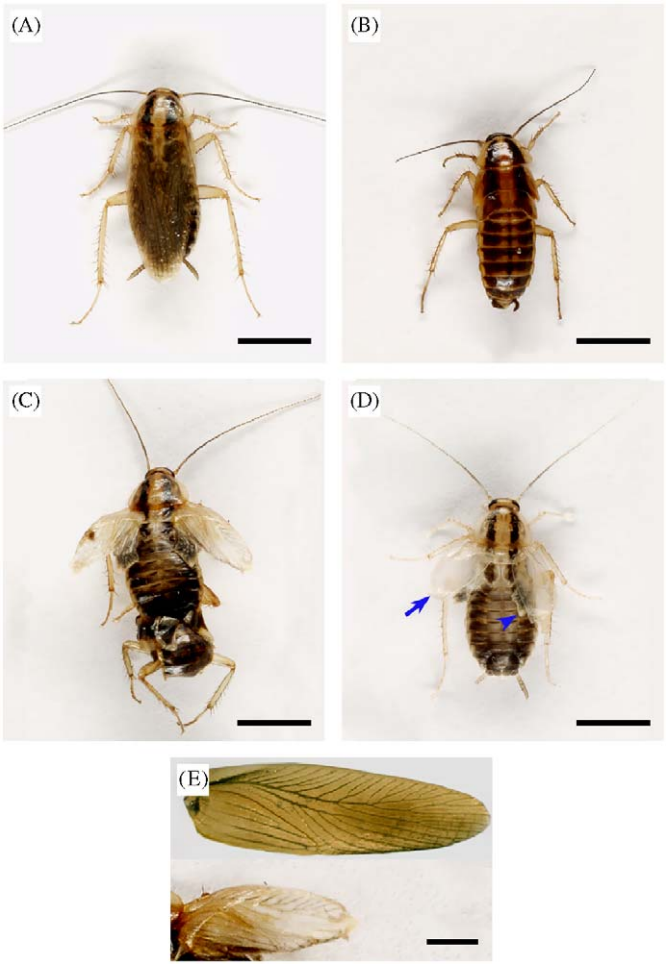

Fig. 4. Effects of BgRXR RNAi on the imaginal molt of *B. germanica*. Freshly emerged sixth instar female nymphs were treated with dsBgRXR or with dsControl and were analyzed at molting, 8 days later. (A) dsControl treated specimen 1 day after the imaginal molt, showing a normal adult appearance. (B) dsBgRXR treated nymph of the same age that arrested at the molting process. (C) dsBgRXR treated nymph that started the molting process but did not complete it successfully. (D) dsBgRXR treated nymph 1 day after successful imaginal molt but showing severe defects of wing expansion in both the forewings (arrow) and hindwings (arrowhead). (E) Forewing of a dsControl treated specimen 1 day after the imaginal molt (upper picture) and from ecdysed dsBgRXR specimen showing defective tanning and extension (lower picture). Scale bars: 5 mm in A–D; 2 mm in E.

after 24 h, became arrested and died within the next 24–48 h (Fig. 4B). Moreover, 37% started the ecdysis process, but failed to shed the exuvia completely (Fig. 4C). Finally, 12% ecdysed properly into adults, although all of them were unable to extend their fore- and hindwings (Fig. 4D) and were significantly lighter (17%) with respect to dsControl-treated nymphs (weight dsControls: 62.3 ± 1.46 mg; mean weight dsBgRXRs: 51.05 ± 2.59 mg; $n = 6$ and 11, respectively, t-test, $p \leqslant 0.001$). It was noteworthy that the adult forewings of all the ecdysed or partly ecdysed animals were not properly tanned, whereas all the other structures were perfectly tanned (Fig. 4C–E). All dsControl-treated nymphs ($n = 24$) ecdysed and tanned correctly (Fig. 4A).

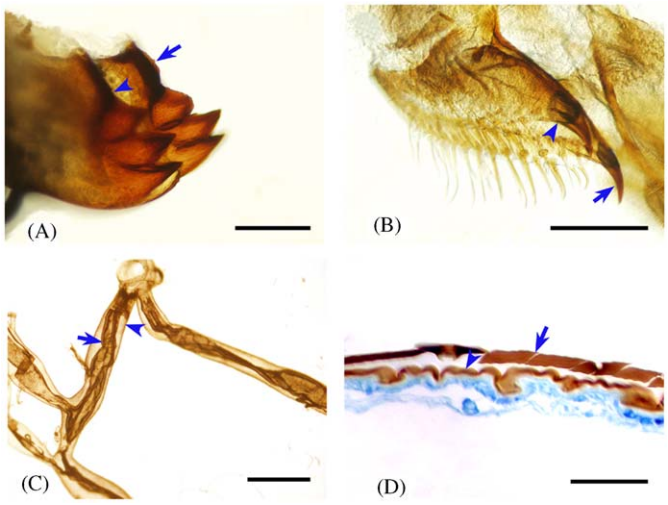

Fig. 5. Phenotype of arrested BgRXR knockdowns of *B. germanica*, showing duplication of cuticular structures, like (A) double mandible, (B) double laciniae and (C) double trachea of the same specimen. (D) Histological section of the cuticle from dsBgRXR nymph knockdowns also showing double structures. Structures of nymphal origin are indicated with an arrow, whereas structures of adult origin are indicated with an arrowhead. Scale bars: 500 μm in (A–C); 50 μm in (D).

Remarkably, the arrested dsBgRXR-treated nymphs exhibited duplicated structures of ectodermic origin. For example, two pairs of mandibles and laciniae were clearly visible, (Fig. 5A and B), they displayed a double tracheal system (Fig. 5C), and the foregut and hindgut portions of the digestive tract were similarly duplicated (not shown). Histological examination of the abdominal cuticle showed that arrested BgRXR knockdowns had two superimposed cuticle layers (Fig. 5D). The new (adult) cuticle presented the endocuticle and exocuticle layers well formed, whereas the old (nymphal) endocuticle was partially digested, with only the exocuticle visible.

4. Discussion

The results presented in this paper demonstrate that *B. germanica* is sensitive to dsRNA-mediated post-transcriptional gene silencing in vivo, either in nymphal or adult stages. The main advantages of this technique are the ease of application, as well as its reliability and specificity of action. Moreover, the high degree of silencing in *BgVg* and *BgRXR* expression in different tissues within the haemocel demonstrates that the RNAi effect is systemic rather than local, and hence generates a whole animal phenotype. Similar disruption of *vitellogenin* gene function by RNAi has been obtained in the honey bee, *Apis mellifera*, by Amdam et al. (2003) (see also Guidugli et al., 2005).

The main objective of the present study was to use the RNAi in vivo approach to elucidate the role of 20E during nymphal development in *B. germanica*. To this end, we first analyzed the role of BgRXR, one of the nuclear receptors comprising the functional 20E receptor. In *B. germanica*, cDNAs encoding two isoforms of BgRXR had been previously cloned and characterized (Maestro et al., 2005). The results presented here reveal that reduction of BgRXR mRNA levels in last instar nymphs impairs the ecdysis process. The same phenotype is obtained when the levels of BgEcR-A mRNA, the heterodimeric partner of BgRXR, are reduced by RNAi (D.M., J.C., D.M-P. and X.B., unpublished results). BgRXR and BgEcR-A knockdowns arrest at the same time and exhibit the same set of molting defects. This result supports the contention that in hemimetabolous insects, EcR and RXR/USP function together as the functional 20E receptor in vivo, at least during the molting process.

The arrested dsBgRXR-treated nymphs are reminiscent of the "double mouthooks" phenotype obtained in *D. melanogaster*, which is characterized by the duplication of cuticular structures. In *D. melanogaster*, the double mouthooks phenotype is typical of mutants for genes involved in steroidogenesis, such as *dare* (Freeman et al., 1999), *molting defective* (Neubueser et al., 2005) or *ecdysoneless* (Gaziova et al., 2004), and with genes related with the 20E-triggered cascade of transcription factors, including EcR-B1 and EcR-B2 (Schubiger et al., 1998; Li and Bender, 2000), *USP* (Li and Bender, 2000) and *βFTZ-F1* (Yamada et al., 2000), or with the nuclear receptor interacting protein *rigor mortis* (Gates et al., 2004). The coincidence between the "double mouthooks" phenotype with the arrested phenotype of *B. germanica* suggest that the latter may be the combined result of a dramatic reduction in BgRXR levels and an alteration in the haemolymph ecdysteroid titer. Indeed, in BgEcR-A knockdown nymphs, there is an overall reduction in the levels of circulating ecdysteroids, while the phenotypes obtained are similar to those of BgRXR knockdowns (D.M., J.C., D.M-P. and X.B., unpublished results).

The fact that dsBgRXR nymphs arrest although correctly synthesizing the new endo- and exocuticle layers and also digesting the old cuticle, indicates that the molting problem resides in the shedding of the nymphal cuticle, that is, in the ecdysis behaviour. This behaviour is a sequential event of precise body contractions that is triggered and controlled by a number of peptides synthesized and released from the CNS and from highly specialized tracheal cells, called Inka cells (for an extended review see Zitnan and Adams, 2005). The sharp increase and decline of circulating ecdysteroids are crucial for the correct timing of the synthesis and release of these peptides. For example, high ecdysteroids levels induce the expression of the *ecdysis triggering hormone (eth)* gene, which leads to an increase in the production of ETH itself and the pre-ecdysis triggering hormone (PETH) in Inka cells. These two peptides activate the pre-ecdysis and ecdysis behaviours. Moreover, the rapid decline in the ecdysteroid titer at the end of the instar leads to the release to the haemolymph of peptides either from the CNS, such as the eclosion hormone (EH) and from Inka cells (PETH and ETH). Finally, EH induces the release of the crustacean cardioactive peptide (CCAP) from neurosecretory cells in

the ventral nerve cord which causes the onset of the ecdysis behaviour (for an extended review see Zitnan and Adams, 2005).

Since *BgRXR* silenced nymphs formed a correct new cuticle, it is clear that the ecdysteroid levels has risen and begun to decline. However, the incapability to carry out the ecdysis properly, suggest that BgRXR is involved in the regulation and/or the sensing of the rapid decline of circulating ecdysteroids at the end of the nymphal instar that normally leads to the release of the peptides (EH, PETH, ETH) that control the ecdysis behaviour.

Finally, it is interesting to note that all of the dsBgRXR knockdowns that molted into adults (12%) failed to extend their wings correctly, both the fore- and the hindwings (Fig. 4D). Moreover, in all cases, the forewings of these animals did not tan properly, as the rest of the body did (Fig. 4E). In *D. melanogaster* it has been shown that wing spreading and tanning are controlled by two neuropeptides, CCAP and bursicon (Park et al., 2003; Dewey et al., 2004). Our results suggest that the release of CCAP and/or bursicon is altered at the end of the ecdysis behaviour in BgRXR knockdowns and as a consequence wing extension and tanning are also affected.

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