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The nuclear hormone receptor BgE75 links molting and developmental progression in the direct-developing insect *Blattella germanica*

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Abstract

Ecdysteroid hormones regulate key developmental processes throughout the life cycle of insects. 20-Hydroxyecdysone (20E) acts upon binding to a heterodimeric receptor formed by the nuclear receptors EcR and USP. The receptor, once 20E bounds to it, elicits cascades of gene expression that mediate and amplify the hormonal signal. The molecular characterization of the 20E-mediated hierarchy of transcription factors has been analyzed in detail in holometabolous insects, especially in *Drosophila melanogaster*, but rarely in more basal hemimetabolous species. Using the hemimetabolous species *Blattella germanica* (German cockroach) as model, we have cloned and characterized five isoforms of *B. germanica* E75, a member of the nuclear receptor family participating in the 20E-triggered genetic hierarchy. The five isoforms present characteristic expression patterns during embryo and nymphal development, and experiments in vitro with fat body tissue have shown that the five isoforms display specific 20E responsiveness. RNAi experiments in vivo during the penultimate and last nymphal instars of *B. germanica* revealed that BgE75 is required for successfully complete nymphal–nymphal and nymphal–adult transitions. Detailed analysis of knockdown specimens during the last nymphal instar showed that BgE75 is required for the rise of circulating ecdysteroids that occurs towards the end of the instar. The main cause of ecdysteroid deficiency in BgE75 knockdowns is the premature stage-specific degeneration of the prothoracic gland. As a consequence, BgE75 knockdown nymphs do not molt, live for up to 90 days and start the adult developmental program properly, in spite of remaining as nymphs from a morphological point of view. Finally, RNAi of specific isoforms during the last nymphal instar of *B. germanica* has showed that they are functionally redundant. Furthermore, it also revealed the occurrence of a complex regulatory relationship among BgE75 isoforms, which is responsible of their sequential expression.

Keywords: Nuclear receptor; *Blattella germanica*; 20-Hydroxyecdysone; Prothoracic gland; RNAi; E75

Introduction

Post-embryonic development in insects is mainly under the control of ecdysteroid hormones, which, among other processes, regulate the transitions between different life stages. In holometabolous species, those presenting complete metamorphosis such as *Drosophila melanogaster*, pulses of 20-hydroxyecdysone (20E, the biologically active form of ecdysteroids) at the end of each larval instar, trigger the molt to the next one, whereas another pulse of 20E at the end of the last larval instar signals the onset of pupariation. Moreover, the presence of 20E during metamorphosis induces the destruction of larval tissues and the formation of the adult body plan (Thummel, 1995). From a molecular point of view, 20E acts upon binding to a heterodimer of two members of the nuclear receptor superfamily, the ecdysone receptor (EcR) and the RXR-ortholog, ultraspiracle (USP) (Yao et al., 1992). The activated receptor elicits cascades of gene expression that mediate and amplify the ecdysteroidal signal. Molecular characterization of several genes that take place in this cascade revealed that they encode transcription factors, such as *E74*, *Broad* and a number of nuclear receptors (reviewed in King-Jones and Thummel, 2005; Riddiford et al., 2000; Thummel, 1995).

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Conversely, characterization of the mechanisms of 20E action operating in hemimetabolous, direct-developing insects is practically nonexistent, with only few exceptions (e.g., Erezyilmaz et al., 2006). In hemimetabolous insects, growth and maturation occur simultaneously throughout successively nymphal stages. In this sense, hemimetabolous development is relatively similar to that of vertebrates. Recently, the German cockroach *Blattella germanica* has been used as model to study this type of development. To analyze how the 20E-triggered genetic hierarchy of transcription factors controls development of *B. germanica*, we have firstly identified the 20E heterodimeric receptor, namely one isoform of EcR, BgEcR-A and two isoforms of RXR/USP, BgRXR-S and BgRXR-L (Cruz et al., 2006; Maestro et al., 2005), as well as three isoforms of the early–late nuclear receptor HR3, namely BgHR3-A, BgHR-B_1 and BgHR3-B_2 (Cruz et al., 2007). Double-stranded RNA (dsRNA)-mediated RNA interference (RNAi) in vivo of BgEcR-A and BgRXR resulted in significant molting impairment. Knockdown nymphs arrested development just before ecdysis and exhibited the typical "double mouthhooks" phenotype characterized by the occurrence of duplicated structures of ectodermic origin, such as mouthparts, hypopharynge, tracheal system and cuticle layers (Cruz et al., 2006; Martin et al., 2006). In a step forward to understand the role of 20E in the development of hemimetabolous insects, we have now cloned and characterized E75, an early gene belonging to the nuclear hormone receptor superfamily in *B. germanica*.

In *D. melanogaster*, the 20E-dependent *E75* gene (*Eip75B* gene in the flybase NR1D3) codes three splice variants, E75A, E75B and E75C, which differ in their N-terminal regions (Segraves and Hogness, 1990). Recently, a fourth isoform, E75D, has been characterized (Dubrovskaya et al., 2004). Mutational analysis of all isoforms has shown that they are required for embryo viability (Bialecki et al., 2002). Conversely, isoform-specific mutants developed until later stages: E75B mutants were totally viable, E75C mutants arrested development as pharate adults or soon after adult emergence, whereas E75A mutants arrested development as first, second or third instar larvae, as well as during early pupae and pharate adults (Bialecki et al., 2002). In addition to *D. melanogaster*, E75 orthologs have been also identified in other holometabolous species, such as the dipteran *Aedes aegypti* (Pierceall et al., 1999), the lepidopterans *Manduca sexta* (Dubrovskaya et al., 2004; Segraves and Woldin, 1993; Zhou et al., 1998), *Bombyx mori* (Swevers et al., 2002), *Galleria mellonella* (Jindra et al., 1994), *Choristoneura fumiferana* (Palli et al., 1997) and *Plodia interpunctella* (Siaussat et al., 2004) and the hymenopteran *Apis mellifera* (Paul et al., 2006).

In this paper, we report the cloning of five isoforms of E75 in *B. germanica*, named BgE75A, -B, -C, -D and -E. In addition, we have examined the developmental expression of the five isoforms during embryo development and throughout nymphal stages, as well as their 20E-responsiveness. Furthermore, we have analyzed the role of *BgE75* during nymphal development by using an RNAi in vivo approach that allowed us to decrease simultaneously the expression of the five E75 isoforms during the penultimate and last nymphal instars. Regarding the last nymphal instar, we have showed that BgE75 knockdown nymphs are ecdysteroid deficient mainly due to the premature degeneration of the prothoracic gland, the tissue responsible for the synthesis of ecdysteroids. Consequently, these specimens do not molt into adults although they start the adult developmental program properly, indicating that BgE75 links molting with developmental progression. On the other hand, the analysis of penultimate nymphal instar knockdowns has showed that BgE75 is also required for the nymphal–nymphal molt, although the prothoracic glands of these specimens do not show any sign of precocious degeneration. Finally, isoform-specific interference of BgE75 during the last nymphal instar has revealed that they are functionally redundant and that the sequential expression of BgE75 isoforms is achieved by a complex regulatory relationship among them.

Experimental procedures

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 and tissue sampling were carried out on carbon dioxide-anesthetized specimens.

Cloning of BgE75 cDNAs

Degenerate primers based on the DNA binding domain (DBD) and ligand binding domain (LBD) of E75 insect homologs were used to obtain a *B. germanica* homolog cDNA fragment by RT-PCR. The first PCR amplification was carried out using as a template cDNA generated by reverse transcription from polyA$^+$ RNA from 20E-treated UM-BGE-1 cells (derived from early embryos of *B. germanica*), as previously described (Cruz et al., 2006; Maestro et al., 2005). The primers used were as follows: forward (BgE75F1), 5′-TGYGARGGNTGYAARGG-3′ and reverse (BgE75R1), 5′-GTRAAYTTRTCRTCYTG-3′. A second PCR was carried out using a nested reverse primer BgE75R2: 5′-GCYTTYTCNCKYTTNGG-3′ and BgE75F1. The 198-bp amplified fragment was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. This was followed by 5′- and 3′-RACE (5′- and 3′-RACE System Version 2.0; Invitrogen) to complete the sequence. For 5′-RACE, reverse primers located at the DBD were BgE75R3: 5′-AACCGCACCG-CATCTCTACTCATT-3′, and the nested BgE75R4: 5′-ATGGAGCACTG-TTGGTTCTTGGTA-3′. For 3′-RACE, the forward primer located at the DBD was BgE75F2: 5′-TACCAAGAACCAACAGTGCTCCAT-3′. As templates, we used cDNA generated from UM-BGE-1 cells as well as different nymphal tissues (prothoracic gland, fat body and epidermis). All PCR products obtained were subcloned into the pSTBlue-1 vector (Novagen) and were fully sequenced in both directions. With this strategy, we isolated five different BgE75 isoforms differing from their DBD and A/B domains. Finally, entire BgE75 open reading frames for each of the five isoforms were amplified by PCR using Accutaq polymerase (Sigma), using a specific forward primer for each isoform and a common reverse primer. The five PCR fragments had the expected size and were subcloned into pSTBlue-1 (Novagen) and sequenced.

RT-PCR/Southern blot analyses

RT-PCR followed by Southern blotting with specific probes was used to determine the expression patterns of BgE75. The RNA was obtained from different tissues and synthesis of cDNA was carried out as previously described (Cruz et al., 2003). Primers for BgE75 isoform-specific amplifications were designed to amplify the 5′UTR and A/B domains. Primers for BgEcR-A and BgRXR amplification were those previously described (Cruz et al., 2006; Martin et al., 2006). Furthermore, primers for *B. germanica* vitellogenin (BgVg) amplification were as follows: forward, 5′-TGAAATGCGAAGGAAAGC-CAA-3′ and reverse, 5′-CCTGTCAAGACCTGAAATGTAT-3′; and for HMG-CoA synthase: forward, 5′-GAAGTCTCTTGCTCGCCTCGTC-3′ and reverse,

5′-TTGTGTTCCCTCGTCTCCATCGT-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′. cDNA samples were subjected to PCR with a number of cycles within the linear range of amplification for each transcript depending on the tissue and physiological stage, as previously described (Cruz et al., 2006, 2007). 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 labeled with fluorescein, using the Gene Images random prime-labeling 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.

Incubation of epidermis/fat body in vitro

Abdominal tergites with epidermis and adhering fat body tissue were dissected from sixth instar female nymphs and incubated in 1 ml of Grace's medium, with L-glutamine and without insect hemolymph (Sigma) at 30 °C in the dark, as described in Cruz et al. (2006).

RNA interference

RNAi in vivo in sixth instar nymphs of *B. germanica* was performed as previously described (Martin et al., 2006; Cruz et al., 2007). The regions selected to generate templates via PCR for transcription of the dsRNAs are described in Table 1. A volume of 1 μl of each dsRNA solution (1 μg/μl) was injected into the abdomen of newly emerged fifth or sixth instar female nymphs.

Microscopy, histological analysis and detection of cell death

All dissections of larval tissues were carried out in Ringer's saline. To examine the cuticle layers, a portion of an abdominal sternite was fixed in 4% paraformaldehyde, dehydrated and embedded in paraffin. Cuticle sections (6 μm) were stained with toluidine blue. Ovaries were fixed in 4% paraformaldehyde, rinsed with PBS–0.2% Tween (PBT) and incubated for 10 min in 1 μg/ml DAPI in PBT. After two washes with PBT, the tissues were mounted in Mowiol 4-88 (Calbiochem). All samples were examined with a Zeiss Axiophot microscope, and images were subsequently processed using Adobe Photoshop. SDS–PAGE of hemolymph and ovarian proteins was carried out according to Martin et al. (1995).

To detect cell death in the prothoracic gland, TUNEL assays were performed using the In Situ Cell Death Detection Kit, Fluorescein (Roche), following the manufacturer's instructions. Staged prothoracic glands were fixed in 4% paraformaldehyde in PBS for 30 min, washed in PBST (0.1% Triton–PBS) and permeabilized by incubation in 0.1% Sodium Citrate-0.1% Triton–PBS for 30 min. Samples were rinsed in PBS and incubated in TUNEL reaction mixture for 1 h at 37 °C. Finally, they were mounted in Mowiol 4-88 (Calbiochem) and examined with a Leica confocal microscope.

Table 1
Features of the dsRNAs designed to specifically interfere the five splicing variants of BgE75 found in *B. germanica*

dsRNA	DNA fragment	Region targeted
dsBgE75-1	612 bp	Common Hinge and LBD (nucleotide 427 to 1038 in BgE75A; accession number: AM238653)
dsBgE75-2	613 bp	Common F region (nucleotide 1636 to 2348 in BgE75A; accession number: AM238653)
dsBgE75A	205 bp	5′UTR and A/B region of BgE75A (nucleotides 1 to 205 of BgE75A; accession number: AM238653)
dsBgE75B	163 bp	5′UTR and A/B region of BgE75B (nucleotides 1 to 163 of BgE75B; accession number: AM238654)
dsBgE75C	223 bp	A/B region of BgE75C (nucleotides 1 to 223 of BgE75C; accession number: AM710419)
dsBgE75D	125 bp	5′UTR and A/B region of BgE75D (nucleotides 27 to 151 of BgE75A; accession number: AM710420)
dsBgE75E	131 bp	5′UTR and A/B region of BgE75E (nucleotides 104 to 234 of BgE75A; accession number: AM710421)

Treatments in vivo with ecdysteroids

Sixth instar nymphs interfered for BgE75 were injected at day 6 with 20E (Sigma) at doses of 1, 5, 10 and 20 μg per specimen in 1 μl of Ringer saline with 10% ethanol. Controls received the same volume of solvent.

Quantification of hemolymph ecdysteroids

Hemolymph ecdysteroids were quantified by ELISA following the procedure described by Porcheron et al. (1989) and adapted to *B. germanica* by Pascual et al. (1992) and Romañá et al. (1995). 20E (Sigma) and 20E-acetylcholinesterase (Cayman) were used as standard and enzymatic tracer, respectively. The antiserum (AS 4919, kindly supplied by Prof. P. Porcheron) was used at a dilution of 1/50,000. Absorbances were read at 450 nm, using a Multiscan Plus II Spectrophotometer (Labsystems). The ecdysteroid antiserum used has the same affinity for ecdysone and 20E (Porcheron et al., 1989), but since the standard curve was obtained with the later compound, results are expressed as 20E equivalents.

Incubation of corpora allata and quantification of juvenile hormone synthesis

Individual corpora cardiaca–corpora allata complexes were incubated in 100 μl of TC199 medium (Sigma) containing L-methionine (0.1 mM), Hank's salts, HEPES (20 mM) plus Ficoll (20 mg/ml), to which L-[^{3}H-methyl] methionine (Amersham) had been added to achieve a final specific activity of 7.4 Gbq/mmol. Juvenile hormone (JH)-III, the native JH of *B. germanica*, was quantified after 3 h of incubation, according to Piulachs and Couillaud (1992).

Results

Isolation and sequence analysis of five E75 cDNAs from B. germanica

Cloning of E75 cDNAs from *B. germanica* was accomplished by RT-PCR using degenerate primers designed on the basis of conserved motifs of the DBD and the LBD of insect E75 sequences. Using cDNA from the UM-BGE-1 cell line as a template, a 198-bp PCR fragment was obtained, the sequence of which showed high similarity with reported insect E75 sequences. To obtain full-length cDNAs, we used a combination of 3′-RACE and 5′-RACE methodologies with a set of primers located within the LBD, and different cDNAs generated from UM-BGE-1 cells and several nymphal tissues; i.e., prothoracic gland, fat body and epidermis. Using this strategy, five cDNA sequences of 3.23, 3.10, 3.25, 2.94 and 3.25 kb were obtained. Database BLAST search revealed that they encoded *B. germanica* homologs of the nuclear receptor E75. They were classified as BgE75 isoforms, on the basis of the similarity of their A/B domains as well as of their DBDs in comparison with other insect E75 members. Thus, *B. germanica* isoforms were named BgE75A (accession number: AM238653), BgE75B (accession number: AM238654), BgE75C (accession number: AM710419), BgE75D (accession number: AM710420) and BgE75E (accession number: AM710421).

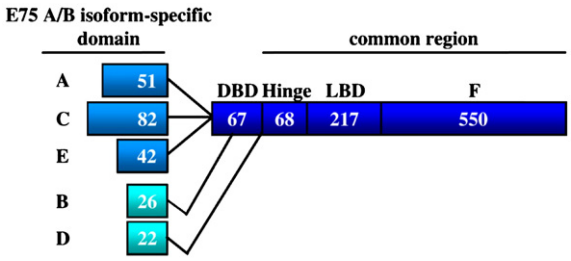

Fig. 1. Domain structure of the five E75 nuclear receptor isoforms identified in *B. germanica*. The number of amino acids of each domain is indicated.

Each BgE75 isoform is characterized by a different N-terminal sequence encoding a specific A/B domain. Three of them, BgE75A, BgE75C and BgE75E, show all the characteristics defining a nuclear hormone receptor, that is, a conserved two zinc-fingered DBD and an LBD, in addition to the A/B domain, hinge region and the F domain in the C-terminus region (King-Jones and Thummel, 2005). Conversely, E75B show only the second of the two zinc-fingers of the DBD, whereas BgE75D lacks the entire DBD (Fig. 1).

Developmental expression of BgE75 in B. germanica

The next step in the characterization of *BgE75* was to establish the developmental expression patterns of the five isoforms during the embryonic development and nymphal stages of *B. germanica*.

B. germanica embryogenesis lasts for 17 days, during which two clear pulses of 20E are detected (Fig. 2, upper panel; data from Maestro et al., 2005). The first one occurs between days 5 and 6, and the second one between days 11 and 15. BgE75A mRNA presented three peaks of expression. Two of them, at days 5–6 and 12–14, took place in correlation with the two ecdysteroid pulses. Remarkably, the first one, at day 2, occurred when the ecdysteroid titer was very low. BgE75B mRNA presented two peaks of expression coinciding with the two ecdysteroid pulses. Furthermore, BgE75C mRNA was detected in 0-day-old embryos, suggesting that it corresponds to maternally inherited transcript. Later, BgE75C is expressed during mid embryogenesis between the two ecdysteroid pulses, that is, between days 6 and 13. BgE75D mRNA was present after the two ecdysteroid pulses, and finally BgE75E mRNA

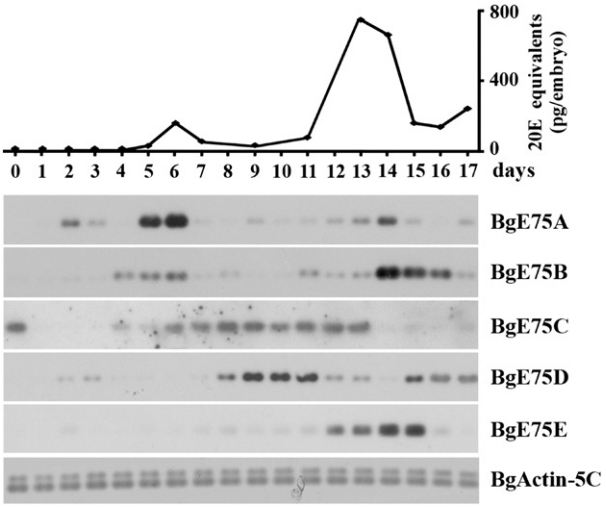

Fig. 2. Expression patterns of BgE75 mRNAs during embryonic development of *B. germanica*. Ecdysteroid levels, in terms of 20E equivalents (upper panel), are from Maestro et al. (2005). Equal amounts of total RNA from staged embryos were analyzed by RT-PCR/Southern blotting using BgE75-specific probes for each isoform (lower panels). BgActin5C levels were used as a reference. The Southern blots shown are representative of three replicates.

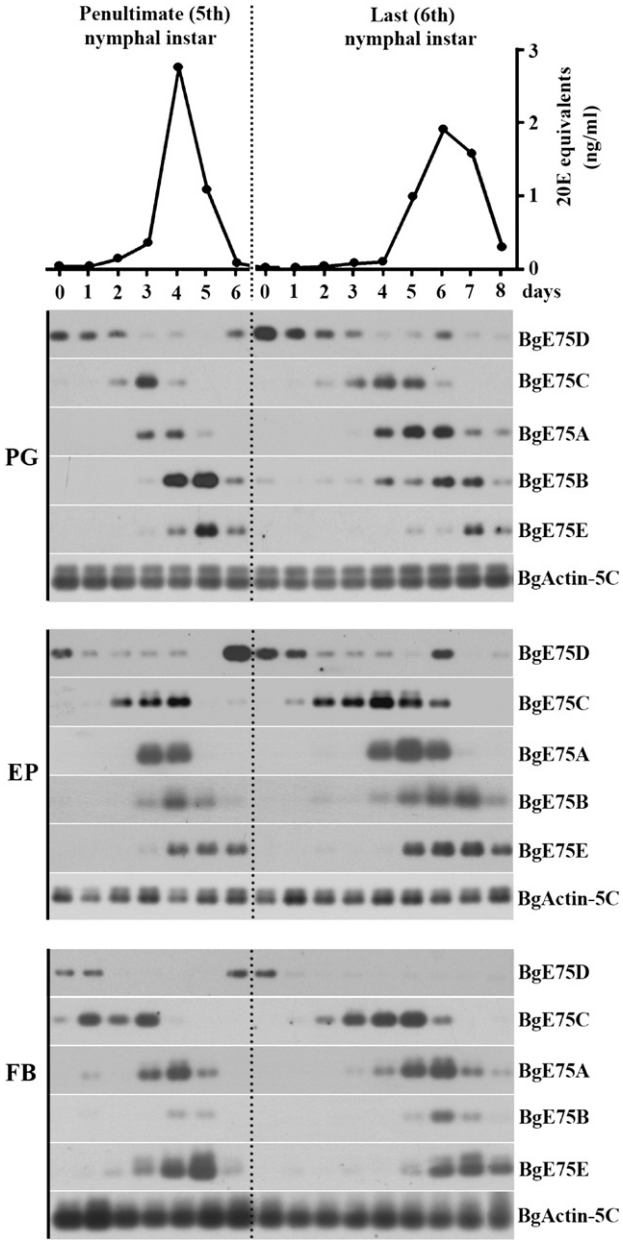

Fig. 3. Expression patterns of BgE75 mRNAs during the last two nymphal instars of *B. germanica*. Ecdysteroid levels in terms of 20E equivalents (upper panel) are from Cruz et al. (2003). mRNA levels were analyzed in the prothoracic gland (PG), epidermis (Ep) and fat body (FB) from staged nymphs by RT-PCR/Southern blotting using BgE75-specific probes for each isoform (lower panels). BgActin5C levels were used as a reference. The Southern blots shown are representative of three replicates.

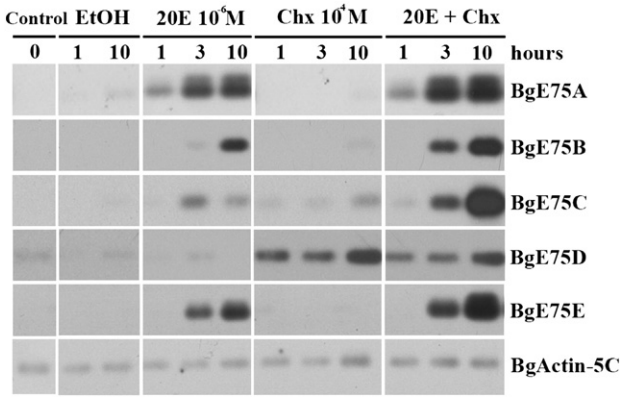

Fig. 4. Effect of 20-hydroxyecdysone (20E) on BgE75 mRNA levels in abdominal tergites with the corresponding epidermis and associated fat body from 1-day-old sixth instar female nymphs of *B. germanica*. Tergites were incubated in vitro in the presence of either $5\times10^{-}6$M 20E; 10^{-4}M of cycloheximide (Chx); or both 20E and Chx for the time indicated. Equal amounts of total RNA from the tissues incubated *in vitro* were analyzed by RT-PCR/Southern blotting using BgE75-specific probes for each isoform. BgActin5C levels were used as a reference. The Southern blots shown are representative of three replicates.

was only expressed in correlation with the second ecdysteroid pulse, between days 12 and 15 (Fig. 2, lower panel).

Transcription of the *BgE75* gene was also analyzed in different tissues during the fifth and sixth nymphal instars of *B. germanica*, encompassing nymphal–nymphal and nymphal–adult transitions. Expression patterns were obtained in the prothoracic gland, the tissue responsible for the synthesis of ecdysteroids during these stages, and in the epidermis, the tissue that synthesizes the new cuticle at the end of each nymphal instar, as well as in the fat body, the main metabolic tissue in insects (Fig. 3). The expression patterns of the five isoforms were similar in the three tissues analyzed. BgE75D was the transcript that appeared earlier at both nymphal instars, although its expression declined rapidly during the first days of each instar. Then, BgE75C appeared, reaching maximal levels of expression just at the rise of circulating ecdysteroids. The levels of BgE75C started to decrease when ecdysteroid levels were higher and became undetectable at the end of each instar. Finally, BgE75A, BgE75B and BgE75E were expressed sequentially from the mid-second-half of each instar, coinciding with the ecdysteroid peak that induces molting.

Effect of 20E on BgE75 expression

The expression patterns of the five BgE75 transcripts suggested to us that 20E was involved in their regulation. Thus, to assess their 20E-responsiveness, we measured BgE75 mRNA levels in abdominal tergites with their epidermis and adhering fat body tissue from 1-day-old sixth instar female nymphs, incubated in vitro for 1 to 10 h in the presence of either 20E (5×10^{-6}M), the protein synthesis inhibitor Chx (10^{-4}M), or both 20E and Chx. Results (Fig. 4) showed that mRNA levels

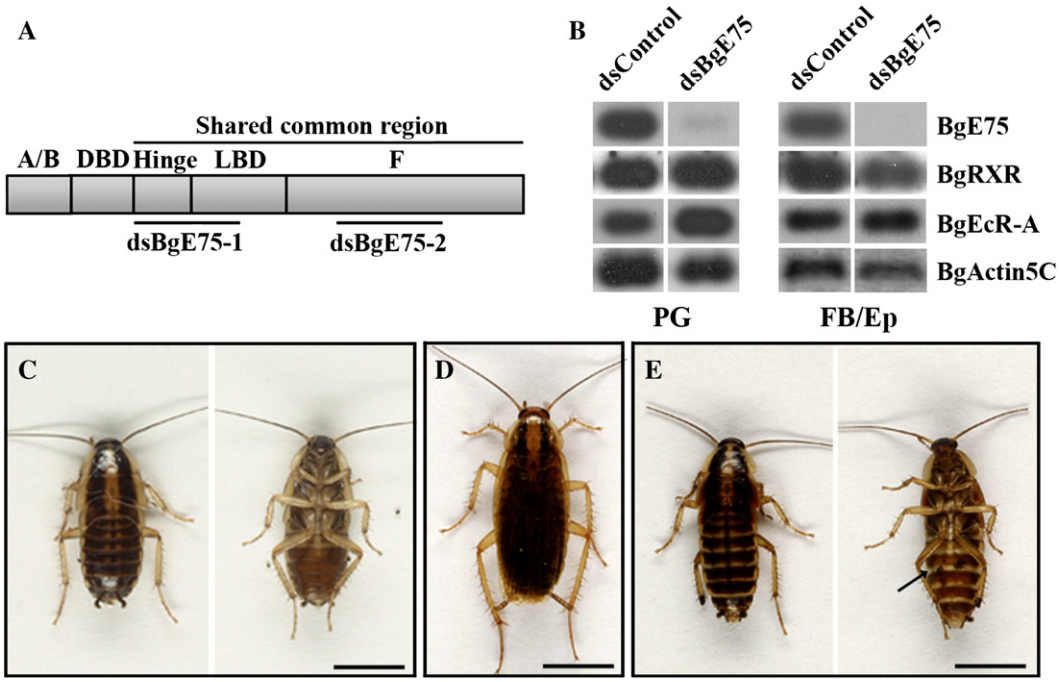

Fig. 5. RNAi *in vivo* of BgE75 in sixth instar female nymphs of *B. germanica*. (A) Scheme of BgE75 organization showing the regions used to generate the dsRNAs. (B) Effects of dsBgE75-1 on mRNA levels of BgE75, BgRXR and BgEcR-A. One microgram of dsBgE75-1 was injected in newly emerged sixth instar female nymphs, and mRNA levels were measured 6 days later in the prothoracic gland (PG) and in the fat body plus epidermis (FB/Ep) by RT-PCR/Southern blotting. Equivalent experiments injecting a non-specific sequence (dsControl) served as negative controls. BgActin5C levels were used as a reference. The Southern blots shown are representative of at least ten replicates. (C–E) Effect of RNAi of BgE75. Newly emerged sixth instar female nymphs were injected with 1 μg of dsBgE75-1 or with dsControl. (C) dsControl-treated nymphs 8 days after the injection, showing the normal nymphal appearance. (D) dsControl-treated specimen 9 days after the injection, that is, 1 day after completing the imaginal molt, showing normal winged adult phenotype. (E) dsBgE75-1-treated nymphs 14 days after the injection, showing the nymphal morphology, with the abdomen clearly expanded (arrow). Scale bar: 0.5 mm.

of BgE75A, BgE75B, BgE75C and BgE75E rapidly increased in response to 20E. The 20E-dependent inductions were even greater when Chx was added to the medium, thus indicating that the effect of 20E upon gene transcription was direct. Incubations in the presence of Chx alone resulted only in a very modest increase in BgE75C mRNA. Conversely, BgE75D mRNA levels were not affected when only 20E was added to the medium, although their levels clearly increased in the presence of Chx, either alone or in combination with 20E.

Effects of RNAi upon BgE75 mRNA levels and molting

We used RNAi in vivo to study the effects of reducing BgE75 expression during nymphal development of *B. germanica*. First, we tried to lower BgE75 expression by injecting a 612-bp dsRNA interfering fragment (dsBgE75-1; Fig. 5A) encompassing the common hinge and LBD regions and hence able to target all isoforms. dsBgE75-1 was injected in the abdomen of freshly ecdysed last instar female nymphs, and BgE75 mRNA levels were determined in the prothoracic gland and in the fat body with adhered epidermis 6 days later. Specimens injected with dsControl were used as negative controls. Results (Fig. 5B) showed that BgE75 mRNA levels decreased substantially in the prothoracic glands and in the epidermis and fat bodies of dsBgE75-1-treated insects compared to controls. Furthermore, mRNA levels of BgEcR-A and BgRXR, the two components of the functional 20E receptor, remained unchanged in dsBgE75-1-treated nymphs, thus indicating that the interference was gene specific (Fig. 5B). The same reduction in BgE75 expression was obtained when a second 613-bp dsRNA targeted to the common F domain (dsBgE75-2; Fig. 5A) was used (data not shown).

Once demonstrated the effectiveness of the dsRNA treatment on *BgE75* expression, we proceeded to the phenotypic analysis of *BgE75* functions by injecting dsBgE75-1 or dsControl to freshly ecdysed sixth instar nymphs. BgE75 knockdown nymphs progressed throughout the instar without any detectable problems. However, at the end of the instar, 92.5% of BgE75 knockdowns ($n=412$) did not molt into adults and remained as viable extended sixth instar nymphs for up to 90 days (Fig. 5E). The same phenotype was obtained when dsBgE75-2 was used (95.1% of extended viable sixth instar nymphs; $n=82$). The BgE75 knockdown nymphs were characterized by continuous feeding (sixth instar nymphs only feed during the first 4 days of the instar), which produced bigger animals with severe abdominal bloating (Fig. 5E, arrow). Conversely, all the

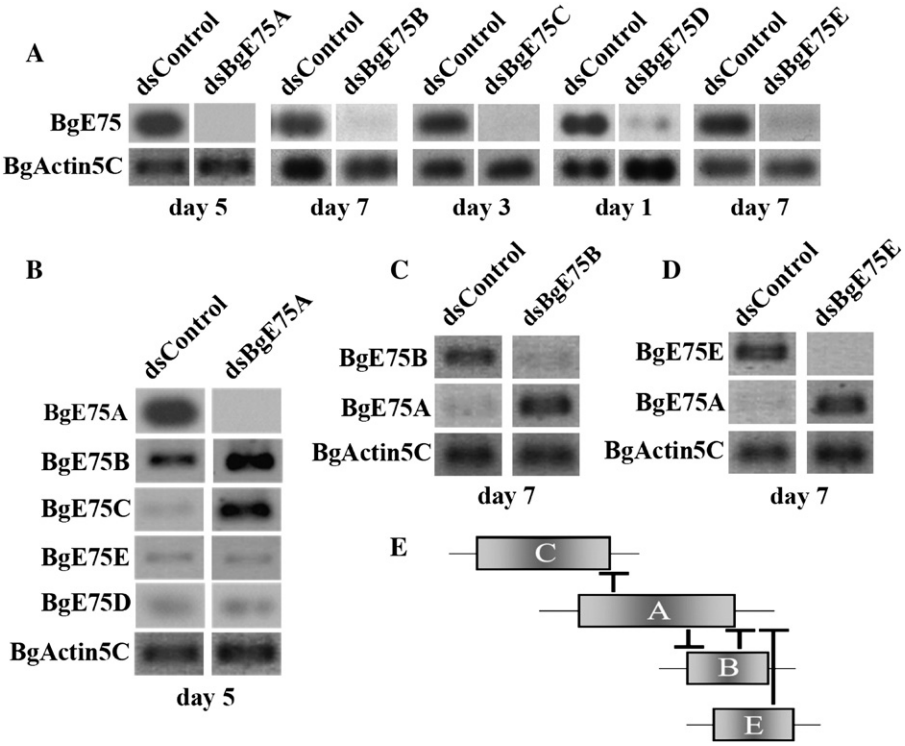

Fig. 6. Isoform-specific knockdown of BgE75 isoforms in sixth instar female nymphs of *B. germanica*. dsRNA (1–5 μg) specific for each BgE75 isoform was injected in newly emerged sixth instar female nymphs, and mRNA levels were studied at the days indicated (which are those of maximal expression of the respective isoform) in the prothoracic gland. (B) Effect of RNAi of BgE75A on the expression of the other BgE75 isoforms. One microgram of dsBgE75A was injected in newly emerged sixth instar female nymphs, and mRNA levels of different BgE75 isoforms were studied 5 days later. (C, D) Effect of RNAi of BgE75B (C) and BgE75E (D) on the expression of BgE75A. One microgram of dsBgE75B or dsBgE75E was injected in newly emerged sixth instar female nymphs, and mRNA levels of BgE75A were studied 7 days later. In all cases (A–D), mRNA levels of BgE75 isoforms were measured by RT-PCR/Southern blotting using the corresponding specific probes. Equivalent experiments injecting a non-specific sequence (dsControl) served as negative controls. BgActin5C levels were used as a reference. The Southern blots shown are representative of at least ten replicates. (E) Model for BgE75 isoform-specific expression during the sixth nymphal instar of *B. germanica*. The figure summarizes the regulatory interactions described in the text. The length of the boxes represents the duration of the expression of BgE75 isoforms.

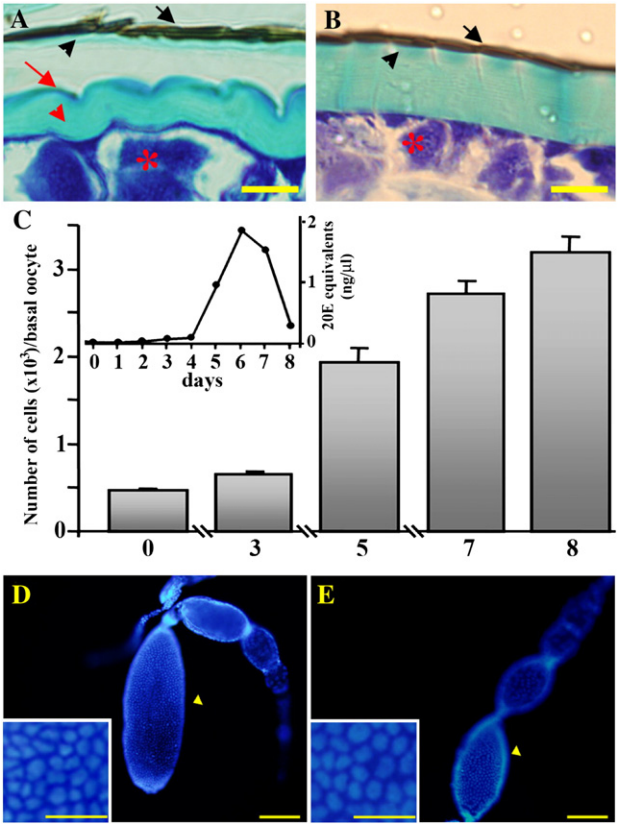

Fig. 7. Effect of RNAi of BgE75 on the epidermis and in the follicular epithelium of the basal oocyte of sixth instar female nymphs of *B. germanica*. Newly emerged sixth instar female nymphs were injected with 1 µg of dsBgE75-1 or with dsControl. Eight days later, histological sections of the cuticle of (A) dsControl and (B) dsBgE75-1-treated nymphs were obtained. Epicuticle (arrows) endocuticle (arrowheads) and epidermis (asterisks) are indicated. Black arrows and arrowheads represent nymphal epi- and endocuticle. Red arrows and arrowheads represent adult epi- and endocuticle. (C) Number of follicular cells in the basal oocyte of staged sixth instar female nymphs. The upper panel shows the hemolymph ecdysteroid levels during the instar, according to Cruz et al., 2003. (D) DAPI-stained ovariole from dsControl 8-day-old sixth instar nymph or from (E) dsBgE75-treated nymph. Arrowheads indicate basal oocytes. Inserts show a detail of the follicular cells that surround the basal oocyte. Scale bars: 10 µm in panels A and B; 100 µm in panels D and E; and 25 µm in insets of panels D and E.

dsControl-treated nymphs ($n=252$) molted normally into adults 8 days after the treatment (Figs. 5C, D).

To analyze the relevance of each isoform in the observed phenotype, we generated isoform-specific dsRNAs targeted to each one of the five isoforms of BgE75 (Table 1). A dose of 1–5 µg of the respective dsRNA injected at the onset of the sixth instar resulted in a remarkably decrease in the corresponding transcript in the prothoracic gland (Fig. 6), fat body and epidermis (data not shown) when analyzed at the day of maximal expression of each isoform. However, all the isoform-specific knockdown nymphs developed normally and molted to the adult stage without any observable defect ($n=95$ for dsBgE75A-treated; $n=57$ for dsBgE75B-treated; $n=87$ for dsBgE75C-treated; $n=44$ for dsBgE75D-treated; $n=50$ for dsBgE75E-treated), thus suggesting that BgE75 isoforms have redundant functions concerning, at least, the last nymphal stage.

To further confirm the redundancy between isoforms, we injected the 10 possible combinations of two dsRNAs of BgE75 in newly ecdysed sixth instar nymphs ($n=15$–25 for each combination). As before, all the resultant double-knockdown nymphs were able to molt properly and did not show any developmental defect. When we injected three different dsRNAs simultaneously, the efficiency of the interference was drastically reduced (data not shown) preventing further phenotypic analysis.

In addition, taking advantage of the single-isoform-specific interference, we characterized the regulatory relationships among BgE75 isoforms by analyzing the effects of the interference of each isoform on the expression of the others. The absence of BgE75A resulted in high levels of BgE75B and BgE75C expression at day 5 (just when both transcripts showed low levels of expression in dsControl-treated nymphs), whereas the expression of BgE75D and BgE75E remained unchanged (Fig. 6B). Furthermore, the down-regulation of BgE75A expression occurring at day 7 was impaired in dsBgE75B- and dsBgE75E-treated nymphs (Figs. 6C, D). Finally, no differences in BgE75 expression were observed in dsBgE75C and dsBgE75D-treated nymphs (data not shown). Taken together, these results allow us to define a complex regulatory network of sequential expression of the different BgE75 isoforms (Fig. 6E).

Characterization of the BgE75 knockdown phenotypes

Then we were interested in characterizing the phenotype of the dsBgE75-1-treated sixth instar nymphs. Firstly, we obtained histological sections of abdomens of dsBgE75-1- and dsControl-treated nymphs 8 days after the treatment, that is, at the onset of the molting process. Whereas in control specimens the adult endo- and exocuticle was already present below the nymphal cuticle (Fig. 7A), BgE75 knockdowns did

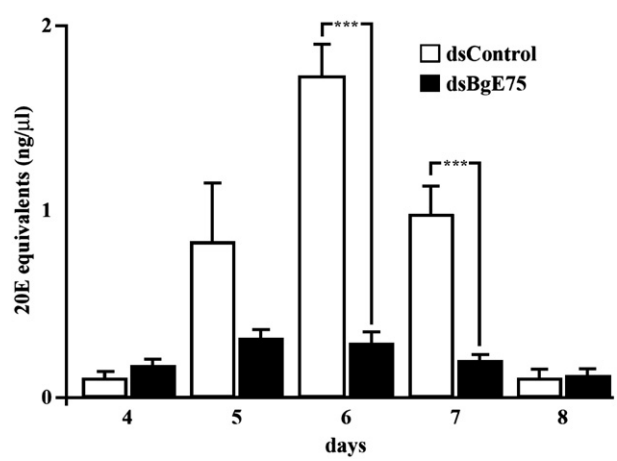

Fig. 8. Effect of RNAi of BgE75 on ecdysteroid levels in *B. germanica*. Hemolymph from dsBgE75-1 and dsControl-treated nymphs was collected at the days indicated of the sixth nymphal instar, and ecdysteroid levels were determined by ELISA. Results are expressed as ng/µl of 20-hydroxyecdysone (20E) equivalents. Vertical bars indicate the SEM ($n=6$–21). Asterisks indicate differences statistically significant at $p \leq 0.0001$ (t-test).

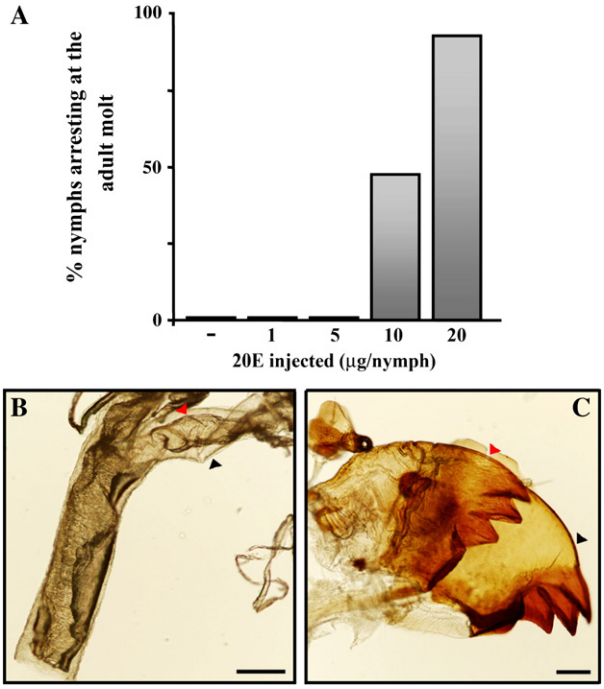

Fig. 9. Effect of 20-hydroxyecdysone (20E) on dsBgE75-1-treated sixth instar nymphs of *B. germanica*. Newly emerged sixth instar female nymphs were injected with 1 µg of dsBgE75-1. Five days later, these nymphs were injected with 1, 5, 10 or 20 µg of 20E in 1 µl of Ringer saline with 10% ethanol, or 1 µl of solvent as a negative control. (A) Percentage of dsBgE75-1-treated nymphs that arrested at the adult molt after injection of 20E or solvent. Ten to twenty nymphs were tested for each dose. (B–C) Arrested nymphs showed duplication of cuticular structures like (B) trachea and (C) mandibles. Black arrowheads indicate nymphal structures, and red arrowheads adult structures. Scale bars: 100 µm in panels B and C.

not present any evidence of adult cuticle deposition (Fig. 7B). In addition, we analyzed the proliferation of the follicle cells surrounding the basal oocytes. In *B. germanica*, the proliferation rate of these cells during the last nymphal instar parallels the increase of circulating ecdysteroids (Cruz et al., 2006), from ca. 500 cells in freshly ecdysed last instar nymphs to ca. 3500 cells just before the imaginal molt (Fig. 7C). Remarkably, at day 8 of the last instar, which would correspond to the end of the instar in a control specimen, BgE75 knockdowns had much fewer follicle cells in the basal oocyte when compared with dsControl-treated nymphs (mean average in dsControl: 3,239 ± 170; in dsE75-1: 1,651 ± 136; $n = 6$ and $n = 10$, respectively, t test, $p \leq 0.00001$), resulting in significantly smaller basal oocytes (Figs. 7D, E).

Ecdysteroid levels in BgE75 knockdowns

The processes affected by the reduction of *BgE75* expression (continuous feeding, impaired molting, adult cuticle synthesis and ovarian follicle cell proliferation) are controlled by increasing levels of circulating ecdysteroids at the end of the nymphal instar of *B. germanica* (Cruz et al., 2006). This suggested to us that the interference of BgE75 would lead to a deficiency of ecdysteroid levels. To test this hypothesis, we measured hemolymph ecdysteroids of dsBgE75-1- and dsCon-

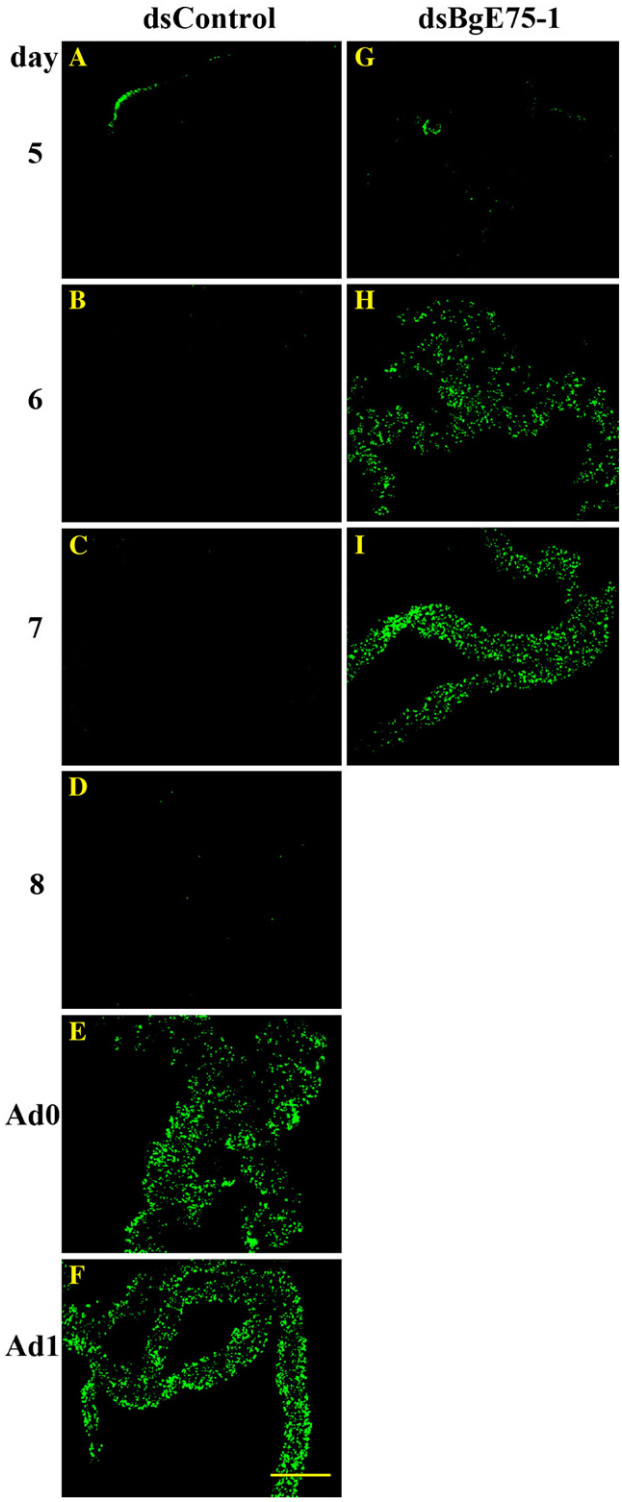

Fig. 10. Effect of RNAi of BgE75 on the timing of prothoracic gland degeneration in *B. germanica*. Newly emerged sixth instar female nymphs were injected with 1 µg of dsBgE75-1 or with dsControl. After 5 days, staged prothoracic glands from dsControl (A–F) and dsBgE75 (G–I)-treated nymphs were collected and labeled with TUNEL to determine DNA fragmentation associated to cell death. In dsControl-treated animals, positive nuclei were only observed in prothoracic glands of newly emerged adults (E–F), whereas in dsBgE75-treated specimens TUNEL positive nuclei were detected from day 6 of the nymphal instar. Scale bar: 200 µm.

trol-treated sixth instar nymphs, between days 4 and 8, when the pulse of circulating ecdysteroids occurs (Cruz et al., 2003). Results (Fig. 8) showed that, whereas ecdysteroid levels in dsControl-treated nymphs presented the acute peak that normally appears between days 5 and 8, those in BgE75-treated specimens were remarkably low during these days.

To further test whether the observed phenotypes were due to a deficiency of ecdysteroids, we tried to rescue the nymphal–adult molt behavior by injecting 20E to 6-day-old sixth instar nymphs that had been treated with dsBgE75-1. Results (Fig. 9A) showed that none of the dsBgE75 knockdowns treated with low doses of 20E (1 and 5 μg/nymph) presented any sign of molting. Conversely, half of the knockdown nymphs treated with 10 μg of 20E and almost all specimens treated with 20 μg of 20E, arrested development at the molting transition (Fig. 9A) and showed duplication of all ectodermal structures, such as mandibles and the tracheal system (Figs. 9B, C).

Role of BgE75 on prothoracic gland degeneration at the nymphal–adult transition

To elucidate the mechanisms underlying the ecdysteroid deficiency showed by the sixth instar BgE75 knockdown nymphs, we examined the morphology of the prothoracic glands. Remarkably, prothoracic glands of knockdown specimens had lost turgidity and cell density by day 8, looking as if they had degenerated. To study the glands in more detail, we used the TUNEL assay to determine DNA

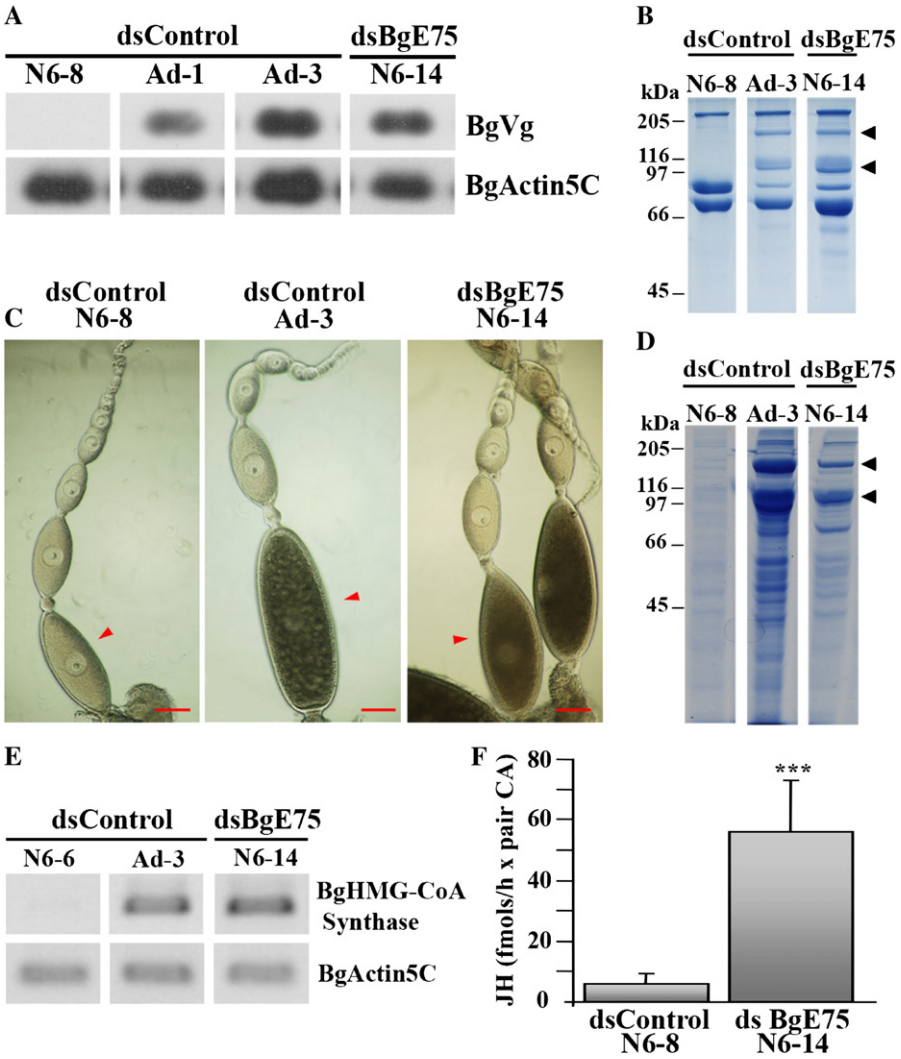

Fig. 11. Onset of the adult developmental program in dsBgE75-1-treated sixth instar nymphs of *B. germanica*. Newly emerged sixth instar female nymphs were injected with 1 μg of dsBgE75-1, and 14 days later they expressed adult-specific markers. (A) Vitellogenin (BgVg) mRNA levels in the fat body of dsBgE75-1-treated nymphs (N6-14) compared to those observed in dsControl-treated adult females on days 1 (Ad-1) and 3 (Ad-3) and nymphs on day 8 (N6-8). BgActin5C levels were used as a reference. The Southern blots are representative of eight replicates. (B) SDS–PAGE of hemolymph from dsControl and dsBgE75-1-treated sixth instar nymphs at the indicated age. Arrowheads indicate BgVg subunits. (C) Ovarioles (scale bars: 125 μm) and (D) SDS–PAGE of 2 μg of ovarian proteins from the specimens shown in panel B. Arrowheads in panel C indicate basal oocytes and in panel D the BgVg subunits. (E) BgHMG-CoA synthase mRNA levels in the corpora allata of dsControl and dsBgE75-1-treated nymphs at indicated ages, analyzed by RT-PCR/Southern blotting. BgActin5C levels were used as a reference. The Southern blots are representative of eight replicates. (F) Rates of JH synthesis by corpora allata incubated in vitro from dsControl and dsBgE75-1-treated nymphs at indicated ages. Vertical bars indicate the SEM. ($n=6$). Asterisks indicate differences statistically significant at $p \leq 0.0001$ (t-test).

fragmentation associated to cell death. As expected, prothoracic glands of dsControl-treated specimens showed no TUNEL labeling throughout the nymphal stage (Figs. 10A–D). Just after the imaginal molt, strong and uniform TUNEL label could be detected as the gland underwent total degeneration by day 1 (Figs. 10E, F). In BgE75 knockdowns, no TUNEL labeling was observed until day 5 of last nymphal stage (Fig. 10G), but, remarkably, strong labeling was observed on day 6 (Fig. 10H), whereas on day 7 the prothoracic gland appeared fully degenerated (Fig. 10I), as in a control adult of day 1 (Fig. 10F).

Onset of the adult developmental program in BgE75 knockdown nymphs

As previously described, sixth instar BgE75 knockdown nymphs that failed to molt continued growing. Interestingly, the basal oocytes of these specimens showed a morphology similar to those from 3-day-old adult females, suggesting that BgE75 knockdown nymphs were expressing genes corresponding to adult development. In this context, we examined the expression pattern of the *vitellogenin* gene (*BgVg*) in the fat body of sixth instar BgE75 knockdown nymphs (Fig. 11A). As expected, *BgVg* was not expressed in control sixth instar nymphs and only began to be expressed 24 h after the imaginal molt (Fig. 11A, lanes 1–2). Remarkably, 14-day-old BgE75 knockdown nymphs expressed *BgVg* in the fat body at levels that were similar to those of 4-day-old adult females (Fig. 11A, compare lane 3 with lane 4). Furthermore, BgVg protein was also detected in the hemolymph and within the growing oocyte of the BgE75 knockdowns, similarly to control adult females (Figs. 11B–D).

Given that activation of *BgVg* gene and incorporation of BgVg into developing oocytes are JH-dependent processes in *B. germanica* (Martin et al., 1995), we then studied whether the corpora allata of BgE75 knockdown nymphs were able to produce JH. Firstly, we assessed that the expression of a key enzyme of JH biosynthesis, 3-hydroxy-3-methyl-glutaryl Coenzyme-A synthase (HMG-CoA synthase) in the corpora allata of control sixth instar nymphs was undetectable (Fig. 11E, lane 1), as expected given the absence of JH production in this stage. Conversely, HMG-CoA synthase mRNA level in the corpora allata of adult specimens was clearly up-regulated (Fig. 11E, lane 2). Interestingly, the corpora allata of BgE75 knockdown nymphs exhibited high levels of HMG-CoA synthase mRNA (Fig. 11E, lane 3). In agreement with that observation, we further assessed that corpora allata of BgE75 knockdown nymphs were able to produce significant amounts of JH when incubated in vitro, at rates comparable to those of a 1- to 3-day-old adult female (Maestro et al., 1994), whereas control sixth instar nymphs practically did not produce JH (Fig. 11F).

Effect of BgE75 RNAi in the penultimate nymphal instar

In order to characterize whether the requirement of BgE75 was exclusive of the nymphal–adult transition or if it was also necessary for the nymphal–nymphal one, 1 μg of dsBgE75-1 was injected into the abdomen of freshly ecdysed fifth (penultimate) instar nymphs. As before, such treatment resulted in a significant decrease in the expression of BgE75 in the prothoracic gland (Fig. 12A) as well as in the fat body and epidermis (data not shown) when compared with the dsControl-treated nymphs. Remarkably, all the dsBgE75-1-treated nymphs ($n=68$) developed normally. However, at the end of the instar, they did not molt and remained as viable fifth instar nymphs for up to 25 days (Fig. 12B). Upon dissection of these specimens, they showed variable degree of duplication of their ectodermic-derived structures. On the other hand, the prothoracic glands of these specimens did not show any sign of degeneration on days 5 and 6, that is, at the time of molting to sixth nymphal instar, when analyzed with TUNEL labeling (Figs. 12C–F).

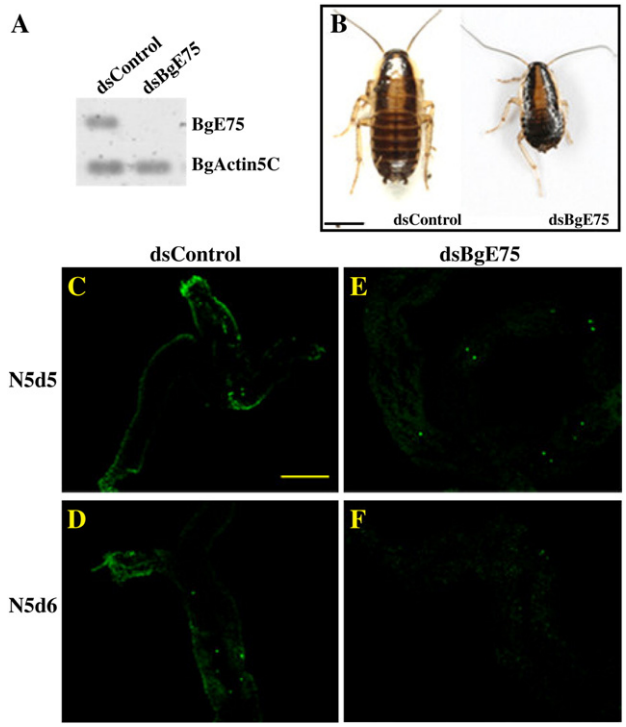

Fig. 12. Effect of RNAi of BgE75 in fifth instar female nymphs of *B. germanica*. (A) Effect of dsBgE75-1 on mRNA level of BgE75. One microgram of dsBgE75-1 was injected in newly emerged fifth instar female nymphs, and mRNA levels were measured 5 days later in the prothoracic gland by RT-PCR/Southern blotting. Equivalent experiment injecting dsControl served as negative controls. BgActin5C levels were used as a reference. The Southern blots shown are representative of at least five replicates. (B) Effect of RNAi of BgE75. Newly emerged fifth instar female nymphs were injected with 1 μg of dsBgE75-1 or with dsControl. dsControl-treated nymphs 7 days after the injection, that is, 1 day after the molting to sixth nymphal instar (left panel). dsBgE75-1-treated nymphs 15 days after the injection, showing the fifth nymphal morphology (right panel). (C–F) Effect of RNAi of BgE75 on the timing of prothoracic gland degeneration in *B. germanica* fifth instar nymphs. Newly emerged fifth instar female nymphs were injected with 1 μg of dsBgE75-1 or with dsControl. After 5–6 days, staged prothoracic glands from dsControl (C, D) and dsBgE75 (E, F)-treated nymphs were collected and labeled with TUNEL. No TUNEL labeling was observed in either dsControl- and dsBgE75-1-treated nymphs. Scale bars: 0.5 mm in panel B; 200 μm in panels C–F.

Discussion

Characteristics of B. germanica BgE75 isoforms

We have described the cloning and characterization of five isoforms of E75, a member of the nuclear hormone receptor superfamily, in the cockroach *B. germanica*. All have a distinctive N-terminal A/B domain and show characteristic expression patterns throughout the life cycle of *B. germanica*. The fact that the nucleotide sequence in all other domains is identical in the five isoforms suggests that they are splice variants generated from the same gene. The classification of the isoforms has been based on the similarity of their A/B regions with described E75 isoforms of other insects. Indeed, multiple isoforms of E75 have only been reported in few holometabolous species. *D. melanogaster* and *M. sexta*, for example, have four isoforms: A, B, C and D (Dubrovskaya et al., 2004; Keshan et al., 2006; Segraves and Hogness, 1990; Segraves and Woldin, 1993; Zhou et al., 1998), whereas *B. mori* and *A. aegypti* have only three: A, B and C (Matsuoka and Fujiwara, 2000; Pierceall et al., 1999; Swevers et al., 2002). The five isoforms described herein are the first to be characterized in a hemimetabolous direct-developing insect, and moreover, a novel type of isoform (BgE75E) is reported. Three of the *B. germanica* isoforms (BgE75A, BgE75C and BgE75E) show the typical five-domains structure, whereas the other two (BgE75B and BgE75D) lack one or both zinc fingers, respectively, in the DBD, which probably renders them incapable of binding to DNA.

Expression patterns and hormonal regulation of BgE75 isoforms

Expression patterns of BgE75 isoforms in *B. germanica* during embryo development (Fig. 2) are remarkably complex when compared to *D. melanogaster*. In the fruit fly, only two isoforms, E75A and E75B, are detected during this stage. E75A is initially expressed 6–8 h after egg laying (AEL) and peaks at 8–12 h AEL, coinciding with the single ecdysteroid pulse that occurs during fruit fly embryogenesis. E75A expression is followed by expression of E75B 8–12 h AEL. Finally, a second peak of E75A mRNA is observed 18–22 h AEL, in the absence of measurable ecdysteroids (Sullivan and Thummel, 2003). Conversely, during the embryogenesis of *B. germanica*, there are two clear ecdysteroid pulses, the first one between days 5 and 6, and the second one in late embryogenesis (Maestro et al., 2005). The occurrence of several ecdysteroid pulses during embryogenesis has been observed in other hemimetabolous insects, such as the locust *Locusta migratoria* (Hoffmann et al., 1980) and the cockroaches *Naphoeta cinerea* (Lanzrein et al., 1985) and *Blaberus craniifer* (Bullière et al., 1979). The dynamic expression of the five BgE75 isoforms throughout embryo development suggests that they are regulating processes at different stages of embryogenesis. Thus, BgE75A, BgE75B and BgE75C (the only transcript of maternal origin) are present during early steps of embryogenesis, which suggests that they could be involved in processes such as blastoderm formation, germ band extension and serosa deposition, which occur from 0 to 3 days of embryo development, and with dorsal closure and the deposition of the pronymphal cuticle, on day 7. Then, BgE75C and BgE75D are highly expressed between the two pulses of ecdysteroids, that is, during pronymphal organogenesis. Finally, all isoforms but BgE75C are expressed concomitantly with the second ecdysteroid pulse, suggesting that they are related with the formation of the first nymphal instar structures, including cuticle deposition. Interestingly, expression of BgE75A at day 2, when no ecdysteroid pulse is detected, suggests that this isoform may be activated in a hormone-independent manner, or that an ecdysteroidal compound that is not detected with our ELISA is regulating their induction.

During nymphal development, the expression of BgE75 isoforms displays a remarkable repetitive and sequential pattern in all tissues analyzed (Fig. 3). Conversely, the expression patterns of E75 isoforms in holometabolous insects, such as *D. melanogaster*, show remarkable variations during post-embryonic development. *D. melanogaster* E75C, for example, is not expressed during larval development, appearing exclusively at the larval-pupal transition. E75A and E75B, on the other hand, are expressed during each larval stage, although their timing of expression varies at each ecdysteroid pulse (Dubrovsky et al., 2004; Sullivan and Thummel, 2003). This difference in the expression patterns of E75 isoforms may reflect the developmental divergence between hemimetabolous and holometabolous insects. In hemimetabolous insects, such as *B. germanica*, growth and maturation occur simultaneously during the successive nymphal instars, whereas in holometabolous species, such as *D. melanogaster*, growth is restricted to larval stages whereas maturation occurs during the pupal stage.

Fat body incubations in vitro have demonstrated that BgE75A, BgE75B, BgE75C and BgE75E are induced by 20E. The induction was rapid and independent of protein synthesis, thus suggesting that these isoforms are early genes in the 20E response hierarchy of *B. germanica*. The 20E effects are similar to those described for E75 isoforms in other insects. *D. melanogaster* E75A, E75B and E75C are induced by 20E in cultured S2 cells (Dubrovsky et al., 2004) or in larval organs (Karim and Thummel, 1992), and also in fat body from adult females of the mosquito *A. aegypti* incubated in vitro (Pierceall et al., 1999). Moreover, expression of E75A and E75C was induced in the ovarian follicle from *B. mori*, when developmentally arrested pupal abdomens were injected with 20E (Swevers et al., 2002). In *M. sexta*, E75A was also induced by 20E in dorsal abdominal epidermis incubated in vitro (Zhou et al., 1998). Conversely, BgE75D did not respond to 20E, but it was rapidly expressed when the fat body was incubated with Chx or 20E plus Chx, which suggests that BgE75D expression is controlled by a 20E-independent repressive factor. This regulation contrasts with those described in *M. sexta*, where E75D was activated by 20E in last larval dorsal abdominal epidermis incubated in vitro and in CH1 cells (Dubrovskaya et al., 2004; Keshan et al., 2006), as well as in *D. melanogaster* S2 cells, where E75D was transiently induced by 20E (Dubrovskaya et al., 2004).

Aside from BgE75D, the expression of the other BgE75 isoforms correlates with the increase in the ecdysteroid titer at the mid-end of each nymphal instar. Interestingly, this expression coincides with those of the three isoforms of BgHR3, another nuclear hormone receptor involved in the cascade of ecdysteroid response (Cruz et al., 2006). A close functional relationship between both receptors has been demonstrated in *D. melanogaster*. White et al. (1997) demonstrated that DHR3 was able to activate the target gene β*ftz-f1* and that E75B impaired this induction by interacting with DHR3. Recently, it has been shown that the interaction of E75 with DHR3 is modulated by the presence of diatomic gases. Thus, *D. melanogaster* E75 contains a heme group within the LBD that acts as a prosthetic group able to bind NO and CO. When CO is bound by E75, it interferes with the ability of this receptor to interact with DHR3, thus making DHR3 able to induce transcription in target genes (Reinking et al., 2005). In *B. germanica*, the similar expression patterns of BgE75 and BgHR3, together with the fact that BgE75 LBD has conserved the two histidine residues that are essential for heme binding in *D. melanogaster* (His 387 and His 171 from BgE75A sequence), suggest that BgE75 can interact with BgHR3, depending on the absence or presence of diatomic gases, during the last part of the nymphal stages.

Role of BgE75 during post-embryonic development

Functional studies on E75 have been reported only in *D. melanogaster*. Homozygote flies with a loss-of-function mutation suppressing all E75 isoforms, $E75^{\Delta 51}$, died as late embryos or during a prolonged first larval instar. Conversely, isoform-specific mutations displayed a range of viability phenotypes. E75B mutants were totally viable and they did not show any detectable phenotypic defect. E75C mutants died as pharate adults or within a few days after the adult eclosion, and E75A mutants died throughout development, from embryo to pharate adult (Bialecki et al., 2002). The results presented herein by using RNAi in vivo during the last nymphal instar of *B. germanica* indicate that *BgE75* is necessary to increase circulating ecdysteroids at the end of the instar (Fig. 8). If the increase does not take place, then a number of developmental processes are severely compromised, such as, follicle cell proliferation of basal oocytes, adult cuticle synthesis and digestion of the nymphal cuticle. BgE75 knockdown nymphs that do not molt into adults continuously feed and can live for up to 80 days before dying. Impairment of the processes seems to be caused by the low levels of circulating ecdysteroids, given that the adult molt is partially rescued by supplying BgE75 knockdowns with 20E (Fig. 9). The permanent nymph phenotype resembles that described in the loss-of-function mutants of all E75 isoforms of *D. melanogaster*, which remain as first instar larvae for over a week, failing to molt to the second instar and dying without any detectable morphological abnormality (Bialecki et al., 2002). Furthermore, a fraction of second instar E75A mutant larvae of *D. melanogaster* also showed a very low ecdysteroid titer during the stage (Bialecki et al., 2002).

Although we cannot rule out the possibility BgE75 regulates the expression of enzymes involved in the ecdysteroidogenic pathway, such those of the "Halloween" group, we have demonstrated that the main cause of the hormonal shortage during the last nymphal instar is the premature degeneration of their prothoracic gland. Conversely to *D. melanogaster*, where the ring gland of E75A mutants appears normal (Bialecki et al., 2002), prothoracic glands of BgE75 knockdowns degenerate from day 6, which is at least 72 h earlier than in control specimens, just at the moment of massive ecdysteroid production. In control *B. germanica*, degeneration of prothoracic gland occurs within 24 h after the imaginal molt and depends on the peak of 20E that takes place at the end of the instar since prothoracic glands of BgEcR-A knockdown sixth instar nymphs that molt into adults do not degenerate (Cruz et al., 2006).

Taken together, the results presented herein show that the ecdysteroid-dependent degeneration of the prothoracic gland is initiated concomitantly with the peak of circulating ecdysteroids occurring at the end of the sixth nymphal instar, and that this induction is repressed by BgE75 until the onset of adult development, when BgE75 expression vanishes. This repressive function of BgE75 concurs with that observed in *D. melanogaster*, where E75 acts as a transcriptional repressor (Jiang et al., 2000; Palanker et al., 2006). Furthermore, the role of E75 on cell death has been scarcely analyzed in *D. melanogaster*. In late prepupal salivary glands, E75A and E75B repress *diap2* (*Drosophila* inhibitor of apoptosis-2) transcription (Jiang et al., 2000). However, *diap2* mutation have shown that it has no role in the regulation of cell death during the development of the fruit fly (Huh et al., 2007; Yin and Thummel, 2005). Furthermore, during *D. melanogaster* oogenesis, E75A and E75B are involved in suppressing or inducing apoptosis of the nurse cells and hence select between degeneration or development of individual egg chambers at stages 8 and 9 (Terashima and Bownes, 2006).

Furthermore, we have demonstrated that BgE75 is not only necessary for the nymphal–adult transition but also for nymphal–nymphal transitions. The fact that dsBgE75-1-treated fifth instar nymphs did not molt to the next instar and live up to 25 days before arresting their development indicates that these animals were also ecdysteroid deficient. However, the arrested specimens show variable degrees of duplication of their ectodermic-derived structures, which suggests that they were able to produce ecdysteroids to some extend. In agreement with these results, the examination of the prothoracic glands indicated that they did not show any sign of precocious degeneration (Fig. 12).

The experiments of isoform-specific interference have shown two remarkable features regarding BgE75 function in *B. germanica*. Firstly, the functional redundancy of the BgE75 isoforms. This is presumed because no effect on the development of sixth instar nymphs was obtained when the expression of each isoform was interfered or even when all possible combinations of two isoforms were knockdown at the same time. In *D. melanogaster*, it has been proposed that E75A and E75C act in a redundant manner when expressed at the same time, that is, at the onset of metamorphosis, and that E75B

could be acting in redundancy with E78B, a homolog of the vertebrate Rev-Erb orphan nuclear receptor (Bialecki et al., 2002). Previously, we had shown in *B. germanica* that three isoforms of the nuclear receptor BgHR3 have redundant functions in the context of ecdysis regulation (Cruz et al., 2007). Secondly, the occurrence of a complex regulatory relationship among BgE75 isoforms, which is responsible of the sequential timing of BgE75 expression during the nymphal instars. Thus, BgE75A controls BgE75C and BgE75B expression at the mid-nymphal instar, whereas BgE75B and BgE75E down-regulate BgE75A expression at the end of the instar (see Figs. 6B–E). These results indicate that inactivation of a single isoform can be functionally replaced by the presence of other isoforms, thus reinforcing the notion of redundancy. Similarly, it has been shown in *D. melanogaster* that E75C is required for sustained transcription of E75B (Bialecki et al., 2002).

BgE75 links molting with developmental progression at the nymphal–adult transition

A remarkably outcome of the reduction in BgE75 expression is that the knockdown last instar nymphs do not molt into adults but continue with the adult-specific developmental program. Several observations support this: (1) there is a clear up-regulation of a key enzyme of the JH biosynthesis, that is, HMG-CoA synthase, in the corpora allata of the BgE75 knockdown nymphs, which start producing significant amounts of JH; (2) the fat body of these animals responds to the production of JH by activating the expression of Vg, the major adult-specific yolk protein precursor; and (3) Vg is incorporated into growing oocytes in an adult-like manner. The ability to start the adult developmental program directly from sixth instar nymphs show that molting process allows the animal growth but do not signal any developmental progression. In *D. melanogaster*, similar results were obtained with E75A mutants, a 20% of which that progress to second larval instar display a heterochronic phenotype characterized by the inability to molt to third instar. However, these mutants survive for several days, showing the developmental program of the third larval instar and finally pupariated although with some delay (Bialecki et al., 2002).

In summary, the results presented demonstrate that in *B. germanica* BgE75 is necessary for successfully completing the different post-embryonic transitions (nymphal–nymphal and nymphal–adult) and that the role of BgE75 in preventing the premature degeneration of the prothoracic gland is stage-specific occurring exclusively during the last nymphal instar.

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