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Nuclear receptor HR4 plays an essential role in the ecdysteroid-triggered gene cascade in the development of the hemimetabolous insect *Blattella germanica*

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ABSTRACT

Despite the differences in the developmental strategies between hemimetabolous and holometabolous insects, a common feature between both types of development is that periodic pulses of the steroid hormone 20-hydroxyecdysone (20E) dictate each developmental transition. Although the molecular action of 20E has been extensively studied in holometabolous insects, data on hemimetabolous is scarce. To address this, we have used the German cockroach *Blattella germanica* to show that 20E signals through a transcriptional cascade of the nuclear hormone receptor-encoding genes *BgE75*, *BgHR3* and *BgFTZ-F1*. Here, we report the isolation and functional characterization of BgHR4, another nuclear receptor involved in this cascade. Expression studies along with tissue incubations and RNAi experiments show that cross-regulation between BgE75 and BgHR3 directs the expression of *BgHR4*. Finally, we have also shown that *BgHR4* is an essential gene required for successfully completing nymphal–nymphal and nymphal–adult transitions, by allowing the appropriate delay in the induction of *BgFTZ-F1*.

1. Introduction

In hemimetabolous insects, growth and maturation occur simultaneously throughout successive nymphal stages until the imaginal molt. This type of development contrasts with that of holometabolous insects, in which an intermediate pupal stage occurs between the juvenile (larval) and adult stages. In holometabolous insects, growth is restricted to larval development, whereas maturation takes place during metamorphosis in the pupal stage. A common feature in both types of development, however, is the central regulatory role exerted by the ecdysteroid hormone 20-hydroxyecdysone (20E). Periodic pulses of 20E trigger nymphal/larval molts and, in holometabolous insects, a pulse of 20E at the end of the last larval instar signals the onset of pupation where it controls the destruction of larval tissues and the formation of the adult structures during metamorphosis (Riddiford, 1993; Thummel, 1995, 2001).

Although there is a detailed understanding of the molecular mechanisms by which 20E regulates the metamorphic process in holometabolous insects, especially in *Drosophila melanogaster* (Thummel, 1996; Riddiford et al., 2000, 2003; King-Jones and Thummel, 2005), little is known about how this hormone operates during the development of hemimetabolous species. Given that holometabolous metamorphosis arose from hemimetabolous ancestors (Sehnal et al., 1996; Truman and Riddiford, 1999; Belles, 2011), it would be interesting to study whether the regulatory mechanisms of 20E action in holometabolous insects are also present in more primitive hemimetabolous insects. We have been using the German cockroach, *Blattella germanica*, to characterize the 20E-triggered hierarchy of transcription factors that responds to, and transduces, the hormonal signal in hemimetabolous insects. In this cockroach, 20E acts upon binding to its heterodimeric receptor formed by the Ecdysone receptor (BgEcR-A) and the retinoid X receptor/ultraspiracle (BgRXR), both expressed in a housekeeping-like pattern throughout nymphal development (Cruz et al., 2006; Martín et al., 2006). In response to 20E binding to BgEcR–BgRXR, several isoforms encoded by three nuclear receptor genes (*BgE75*, *BgHR3* and *BgFTZ-F1*) are sequentially activated and repressed during the second part of the last nymphal instar (Fig. 1) (Cruz et al., 2007; Mané-Padrós et al., 2008). This cascade of nuclear receptors, which is present in most tissues of *B. germanica*, not only controls ecdysteroid biosynthesis and molting during each nymphal instar but also metamorphic-associated processes, such as cell proliferation in wings and in follicular epithelium, and the programmed cell death of the prothoracic gland (Cruz et al., 2006, 2007, 2008; Martín et al., 2006; Mané-Padrós et al., 2008, 2010).

To further complete the characterization of this genetic cascade in *B. germanica*, we have now cloned and characterized a new 20E-dependent nuclear receptor that plays a key role in the regulation of such hierarchy, namely HR4. This nuclear receptor, whose

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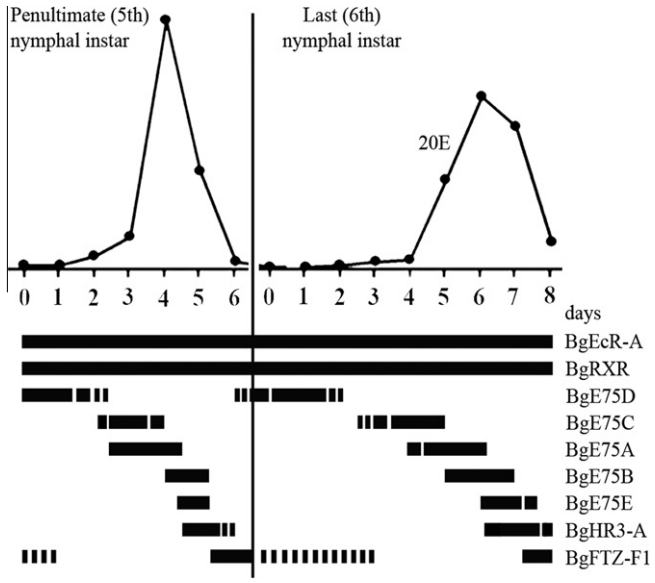

Fig. 1. Circulating ecdysteroid levels and mRNA expression of 20-hydroxyecdysone-dependent nuclear receptors during the last two nymphal instars of *Blattella germanica*. The ecdysteroid (20E) levels are redrawn from Cruz et al. (2003). The diagrams of mRNA expression are based on Cruz et al. (2006, 2007, 2008), Maestro et al. (2005) and Mané-Padrós et al. (2008).

corresponding mammalian ortholog is the transcriptional repressor Germ Cell Nuclear Factor (GCNF) (Fuhrmann et al., 2001; Lan et al., 2002; Sato et al., 2006), has been previously characterized only in holometabolous species, namely the coleopterans *Tenebrio molitor* (Mouillet et al., 1999) and *Tribolium castaneum* (Tan and Palli, 2008), the lepidopterans *Manduca sexta* (Weller et al., 2001) and *Bombyx mori* (Charles et al., 1999) and the dipteran *D. melanogaster* (King-Jones et al., 2005), although loss of function analysis has been carried out only in *T. castaneum* and *D. melanogaster*. In *T. castaneum*, TcHR4 is required for the pupal molt and for successful vitellogenesis and oogenesis during the adult stage (Tan and Palli, 2008; Xu et al., 2010). In *D. melanogaster*, DHR4 exerts a central role coordinating growth and maturation during the last larval instar (King-Jones et al., 2005).

Here, we report the cloning and functional characterization of the HR4 homolog of *B. germanica*, named BgHR4. First, we have examined its developmental expression during embryo development and throughout nymphal stages, as well as the 20E-responsiveness of the gene. Furthermore, by using RNAi in vivo, we have showed that *BgHR4* is a vital gene required for normal development during all the nymphal stages of *B. germanica*. Importantly, we have shown that BgHR4 plays a key role in the 20E-responsive transcriptional cascade by down-regulating the levels of three BgE75 isoforms at the end of the nymphal instar, thus allowing the appropriate delay in the transcriptional up-regulation of the nuclear receptor *BgFTZ-F1*, a master regulator of *B. germanica* development.

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% relative humidity. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens.

2.2. Cloning of BgHR4 cDNA

The *B. germanica* HR4 homologue was obtained by PCR using cDNA template from 20E-treated UM-BGE-1 embryonic cells from *B. germanica*, following the methodology previously described (Maestro et al., 2005; Cruz et al., 2006). Degenerate primers for BgHR4 amplification were, forward (BgHR4-F1): 5′-ATGATVTGYG ARGAYAARGC-3′, and reverse (BgHR4-R1): 5′-TGYTCDATRCAYTT YTTRAA-3′. The amplified fragment (189 bp) 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 extend the sequence. For 5′RACE, reverse primers were (BgHR4-R2): 5′-TACAAGTTGTAAACAGCTCCAGAGTT-3′, the nested (BgHR4-R3): 5′-CGTAGTGCAGACCTGTAGCCTTAT-3′ and the nested (BgHR 4-R4): 5′-TTGCGACCAGATCATCGTCCTTACA-3′. For 3′RACE, forward primer was (BgHR4-F2): 5′-ACCACAAGTCTGATCTCTCAGACAA-3′ and nested (BgHR4-F3): 5′-CACACAAGATTATTAACGCACAAGTG-3′. All PCR products were subcloned into the pSTBlue-1 vector (Novagen, Madison, WI, USA) and sequenced.

2.3. RT-PCR/Southern blot analyses

RT-PCR followed by Southern blotting with specific probes was used to establish the expression patterns of BgHR4. The RNA was obtained from different tissues, and synthesis of cDNA was carried out as previously described (Cruz et al., 2003). Primers used to amplify the different target genes are detailed in Supplemental Table S1. 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, Barcelona, Spain). RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, indicating that there was no genomic contamination.

2.4. 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, Madrid, Spain) at 30 °C in the dark as described (Cruz et al., 2006).

2.5. RNA interference

RNAi in vivo in nymphs of *B. germanica* was performed as previously described (Martín et al., 2006; Cruz et al., 2007). The primers used to generate templates via PCR for transcription of the dsRNAs are detailed in Supplemental Table S2. Control dsRNA consisted of a non-coding sequence from the pSTBlue-1 vector (dsMock) (Cruz et al., 2006). A volume of 1 μl of dsRNA solution (1 μg/μl) was injected into the abdomen of newly emerged fifth or sixth instar female nymphs. In case of coinjection of two dsRNAs, a single injection of 2 μl, consisted of 1 μl of each solution, was applied.

2.6. Microscopy, histological analysis and quantification of hemolymph ecdysteroids

All dissections were carried out in Ringer's saline (9 g/l NaCl, 0.2 g/l KCl, 0.2 g/l $NaHCO_3$, 0.2 g/l $CaCl_2$). Mouthparts and tracheae were directly immersed in 50% glycerol and examined

microscopically. 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. All samples were examined with a Zeiss Axiophot microscope. Hemolymph ecdysteroids were quantified by ELISA as previously described (Romañá et al., 1995). 20E (Sigma) and 20E-acetylcholinesterase (Cayman Chemical, Ann Arbor, MI, USA) were used as a standard and an enzymatic tracer, respectively. The antiserum (Cayman Chemical) was used at a dilution of 1:50,000. Absorbances were read at 450 nm, using a Multiscan Plus II Spectrophotometer (Labsystems, Madrid, Spain). 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.

3. Results

3.1. Cloning and characterization of BgHR4

Cloning of BgHR4 cDNA was accomplished by a RT-PCR approach using degenerate primers designed on the basis of conserved motifs of the DNA binding domain (DBD) of available insect HR4 sequences. Using cDNA from 20E-treated *B. germanica* UM-BGE-1 cells as a template, a 189 bp PCR fragment was obtained, and its sequence was highly similar to insect HR4 sequences. 3′- and 5′-RACE experiments allowed us to extend the sequence in both directions (GenBank accession number JF758869). The protein corresponding to the cDNA obtained shows the domain organization characteristic of a nuclear hormone receptor: a ligand-independent A/B activation domain, followed by the two zinc fingered DBD (C domain) with and adjacent stretch of 32 highly conserved amino acids known as the carboxy-terminal extension (CTE), a hinge region (D domain) and the ligand binding domain (LBD; E domain), which did not contain the putative ligand-independent activation motif, AF-2, and finally a very short F domain (14 amino acids).

The comparison with other HR4 and GCNF sequences (Fig. 2) revealed that the most conserved domains are the DBD (100–98% identity compared with the other insect sequences and 67% compared to human GCNF), the CTE (100% identity with all insect sequences) and the short F domain (100% identity with coleopteran species and 79% with lepidopteran and dipteran species). Conversely, the LBD showed lower identity, 73% compared with coleopteran species and 56–52% compared with lepidopterans and dipterans, and even lower (20%) when compared with the human sequence.

3.2. Developmental expression and 20E-responsiveness of BgHR4

As a first step towards the characterization of BgHR4 function, we obtained expression patterns of *BgHR4* during the embryonic and nymphal development of *B. germanica*. During embryogenesis, *BgHR4* mRNA presented three peaks of expression. Two of them occurred just after 20E pulses, between days 6 and 7 and days 13 and 16, whereas the expression detected at day 2 did not correlate with any detectable ecdysteroid pulse (Fig. 3A).

During nymphal development, the expression pattern of *BgHR4* was obtained in the prothoracic gland (the tissue responsible for the synthesis of ecdysteroids), fat body (the main metabolic tissue) and epidermis (which synthesizes the new cuticle). In all tissues, *BgHR4* mRNA was present during the decline of the ecdysteroid titer at the end of each instar (Fig. 3B).

The expression patterns of *BgHR4* suggested to us that 20E was involved in its regulation. To test this hypothesis, we measured *BgHR4* 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 1–10 h in the presence of either 20E (5×10^{-6} M), the protein synthesis inhibitor cycloheximide (Chx) (10^{-4} M), or both 20E and Chx. As shown in Fig. 3C, a peak of *BgHR4* mRNA appeared 1 h after the addition of 20E. The mRNA then declined to low levels by 10 h. The 20E-dependent induction was greater and sustained during the incubation time when Chx was added to the medium containing 20E. Chx alone did not produce any effect on *BgHR4* expression.

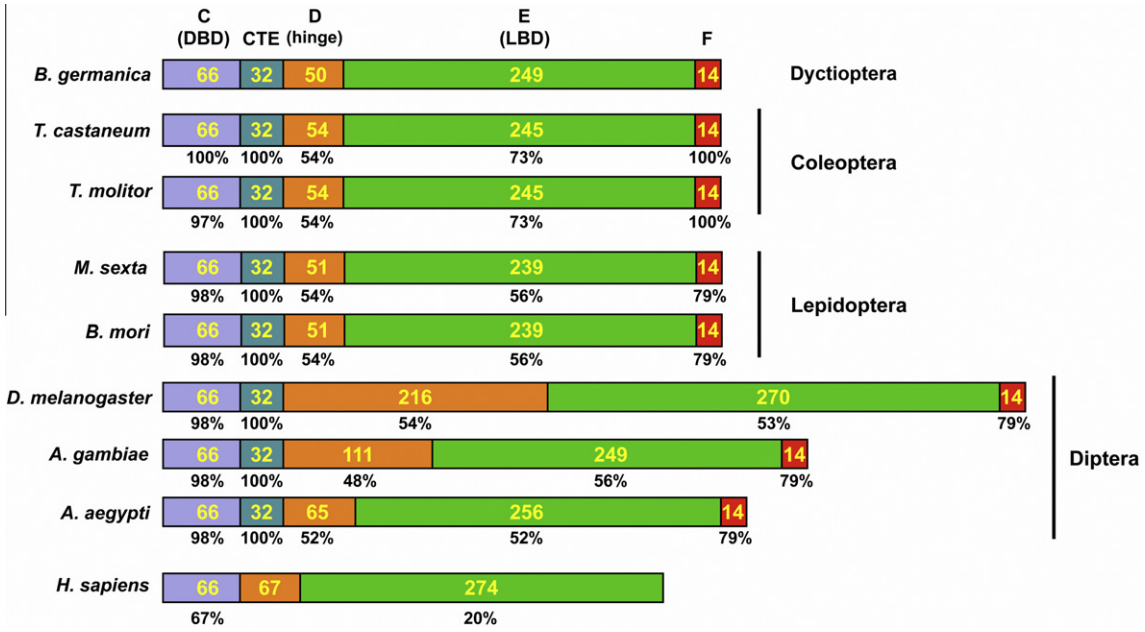

Fig. 2. Domain comparison of *B. germanica* HR4 with other HR4/GCNF nuclear receptors. Letters above BgHR4 indicate functional domains. Numbers within each domain indicate the number of amino acids. The percentages of identity between corresponding domains of BgHR4 and the other orthologs are indicated below each domain. Sequences and species considered are BgHR4 from *B. germanica* (this study), TcHR4 from *Tribolium castaneum* (accession number: XP_974320), TmHR4 from *Tenebrio molitor* (AJ005685), MsHR4 from *Manduca sexta* (AF288088), BmGRF from *Bombyx mori* (AF124981), DmHR4 from *Drosophila melanogaster* (AY971884), AgHR4 from *Anopheles gambiae* (XP_318161), AaHR4 from *Aedes aegypti* (AM773447) and HsGCNF from *Homo sapiens* (AF004291).

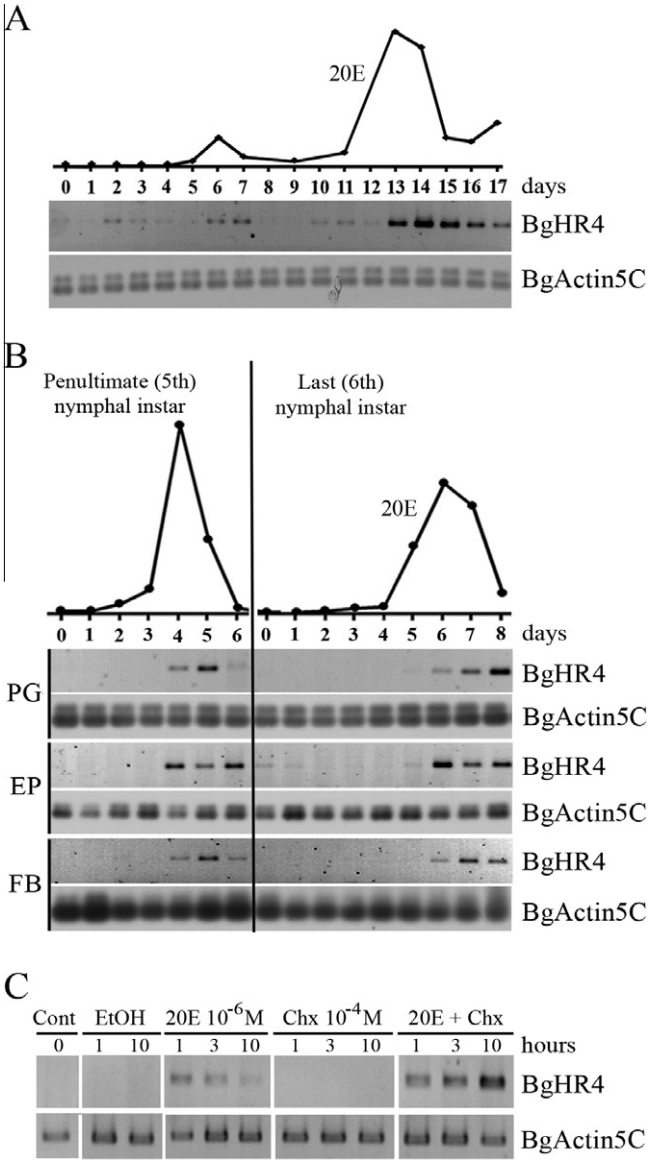

Fig. 3. Expression patterns of *BgHR4* mRNA in *B. germanica* and effect of 20-hydroxyecdysone (20E). (A) Expression pattern of *BgHR4* mRNA during embryo development. Ecdysteroid (20E) levels (upper part) are redrawn from Maestro et al. (2005). Equal amounts of staged embryos were analyzed by RT-PCR/Southern blotting. (B) Expression patterns of *BgHR4* mRNAs during the last two nymphal instars. Ecdysteroid (20E) levels (upper part) are redrawn from Cruz et al. (2003). mRNA levels were analyzed in the prothoracic gland (PG), epidermis (EP) and fat body (FB) by RT-PCR/Southern blotting. (C) Effect of 20E on *BgHR4* mRNA levels in abdominal tergites with corresponding epidermis and associated fat body from 1-day-old sixth instar female nymphs. Tergites were incubated in vitro in the presence of either 5×10^{-6} M of 20E; 10^{-4} M of cycloheximide (Chx); or both 20E and Chx for the time indicated. Equal amounts of total RNA from the tissues were analyzed by RT-PCR/Southern blotting. In all cases, a *BgHR4* specific probe was used for Southern blotting, and *BgActin5C* levels were used as a reference. The Southern blots shown in A–C are representative of five replicates.

3.3. The interplay between nuclear receptors BgHR3 and BgE75 regulates BgHR4 expression

The expression patterns and the experiments in vitro suggest that the activation of *BgHR4* is under a complex regulatory system involving both positive and negative 20E-dependent factors. As the expression of *BgHR3* precedes that of *BgHR4* (Fig. 1; see Cruz et al., 2007), we wondered whether BgHR3 would have a regulatory role on *BgHR4* expression. We tested this possibility with RNAi experiments, by analyzing the expression of *BgHR4* in prothoracic glands of *BgHR3* knockdown nymphs at the end of the last instar. Remarkably, whereas the levels of *BgEcR-A* and *BgRXR* were not affected by the absence of BgHR3, those of *BgHR4* were dramatically reduced (Fig. 4A), clearly indicating that BgHR3 is necessary for *BgHR4* activation.

In contrast, we found an inverse correlation between the expression of *BgE75C* and *BgE75A* and the activation of *BgHR4* (Fig. 1), suggesting a repressive effect of these BgE75 isoforms upon *BgHR4* during the rise of circulating ecdysteroids. Again, we tested this by using RNAi to lower the levels of *BgE75* isoforms. However, given that there is functional redundancy between BgE75 isoforms (Mané-Padrós et al., 2008), we used a dsRNA designed to the common hinge and LBD regions and hence able to target all BgE75 isoforms simultaneously. Confirming the hypothesis, knocking down *BgE75* expression by RNAi resulted in a strong and premature upregulation of *BgHR4* at day 5 of the last instar (Fig. 4B). Interestingly, the reduction of *BgE75* levels also resulted in a strong and premature activation of *BgHR3*, raising the possibility that the repressive effect of BgE75 on *BgHR4* was not direct but rather through the repression of *BgHR3*. We confirmed this relationship by performing RNAi of *BgE75* and *BgHR3* simultaneously and showing that, under these conditions, the premature activation of *BgHR4* was prevented (Fig. 4C).

Collectively, these results indicate that the expression of *BgHR4* is regulated by the interplay of two 20E-dependent nuclear receptors, BgHR3 acting as activator and BgE75 repressing the activation of *BgHR4* indirectly through the negative regulation of *BgHR3* at the onset of the ecdysteroids pulse.

3.4. Disruption of BgHR4 function by RNAi affects nymphal development

To understand the functional relevance of BgHR4 in *B. germanica*, we first silenced it by RNAi in vivo by injecting 1 μg of dsBgHR4-1 (Fig. 5A) in the abdomen of freshly ecdysed last-instar nymphs. As a consequence, mRNA levels of *BgHR4* were dramatically reduced 8 days after the injection, both in the prothoracic gland (Fig. 5B) and in the epidermis and fat body (data not shown). Similar reduction was obtained when a second dsBgHR4-2 was used (Supplemental Fig. 1).

Once demonstrated the effectiveness of the dsRNA treatment, we proceeded to the phenotypic analysis of the BgHR4 knockdown nymphs. Whereas all last instar nymphs treated with dsMock ($n = 89$) molted into adults with the normal developmental timing, those treated with dsBgHR4-1 ($n = 73$), although showed normal appearance and behavior, did not molt at the end of the stage, stopped moving, and finally died 24–48 h after the time when the dsMock nymphs molted into adults (Fig. 5C and D). Furthermore, the arrested nymphs showed duplicated and superimposed ectodermal-derived structures, such as mandibles, maxilla and laciniae in the head, as well as the entire tracheal system (Fig. 5E–G). Moreover, histological sections of the abdomen of these nymphs showed the newly formed adult endocuticle and exocuticle layers below the nymphal exocuticle, thus indicating that the nymphal endocuticle layer had been digested (Fig. 5H). The analysis of the newly synthesized cuticle, including the size and disposition of the abdominal bristles, indicated that it was adult-like (data not shown). The failure of BgHR4 knockdowns to molt was not due to an ecdysteroid deficiency as no differences were found between circulating ecdysteroids of dsBgHR4-1- and dsMock-treated nymphs (Fig. 6). As in the previous experiments, identical phenotypes were obtained when the second dsBgHR4-2 was used (Supplemental Fig. 1).

To ascertain whether the requirement of BgHR4 was restricted to the nymph–adult transition or was necessary for each nymph-nymph transition, we injected 1 μg of dsBgHR4-1 into newly

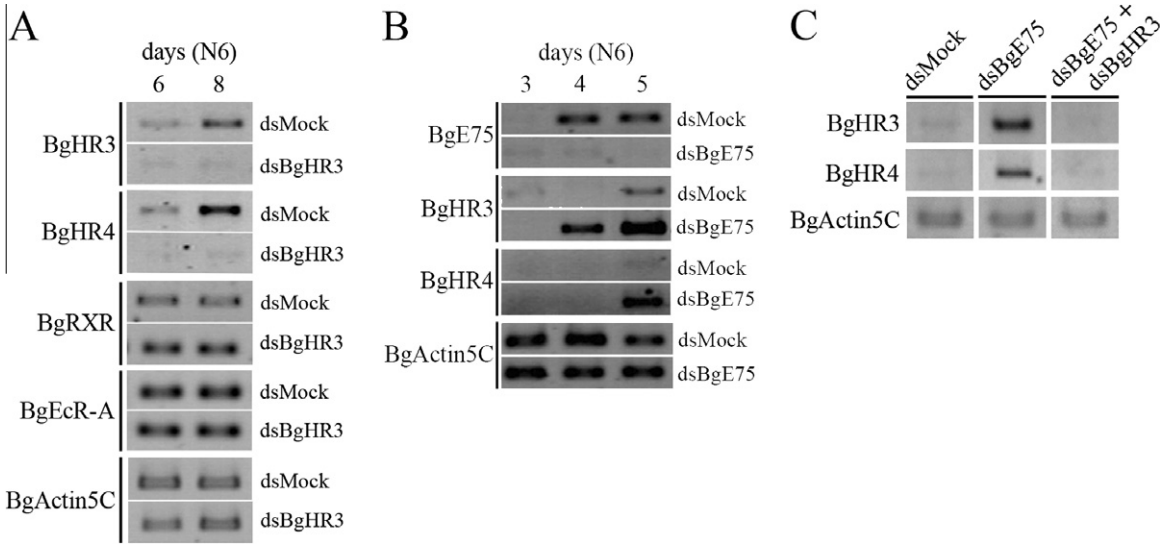

Fig. 4. *BgHR4* expression in *B. germanica* is controlled by the interplay between nuclear receptors BgE75 and BgHR3. (A) Effect of BgHR3-silencing by RNAi on *BgHR4* expression. A dose of 1 μg of dsBgHR3 was injected in newly emerged sixth instar female nymphs, and mRNAs of *BgHR4*, *BgEcR-A* and *BgRXR* were determined in the prothoracic gland 6 and 8 days later. (B) Effect of BgE75-silencing by RNAi on *BgHR4* expression. A dose of 1 μg of dsBgE75 was injected in newly emerged sixth instar female nymphs, and mRNAs of *BgHR4* and *BgHR3* were determined in the prothoracic gland between 3 and 5 days later. (C) Effect of BgE75 or combined BgE75 plus BgHR3 RNAi on the expression of *BgHR4*. Newly emerged sixth instar female nymphs were injected with 1 μg of dsBgE75 or with dsBgE75 plus dsBgHR3 simultaneously (1 μg each) and mRNA of *BgHR4* was determined in the prothoracic gland 5 days later. Equivalent experiments injecting a non-specific sequence (dsMock) served as negative control. In all cases, mRNA levels of all nuclear receptors were determined by RT-PCR/Southern blotting using the corresponding specific probes. *BgActin5C* levels were used as a reference. The Southern blots shown are representative of 10 replicates.

emerged fifth (penultimate) and fourth instar nymphs. As in the last instar nymphs, 99% of the BgHR4 fifth nymphal instar knockdowns ($n = 35$) and 98% of fourth nymphal instar knockdowns ($n = 41$) were unable to complete the molting process and arrested development with duplicated ectodermal structures. The whole data thus indicate that BgHR4 is required for correct molting throughout the postembryonic development of *B. germanica*.

3.5. BgHR4 represses the 20E-dependent cascade at the end of the nymphal instar

The expression pattern of *BgHR4* and the phenotype of BgHR4 knockdown nymphs indicate that this gene plays a critical role in the 20E-regulated transcription at the end of the instar. As molting inhibition in BgHR4 knockdown nymphs is not due to ecdysteroid deficiency, we wondered whether this nuclear receptor played an essential role in the regulation of the transcriptional response to the hormone. In this context, it is worth noting that the phenotypic effects observed in BgHR4 knockdown nymphs are indistinguishable from those seen in BgHR3 and BgFTZ-F1 knockdowns (Cruz et al., 2007, 2008), thus suggesting functional relationships between these three nuclear receptors. In *B. germanica*, the end of each nymphal instar is characterized by the down-regulation of the 20E-dependent nuclear receptors *BgE75A*, *BgE75B* and *BgE75E*, and the strong up-regulation of *BgFTZ-F1* (Cruz et al., 2008; Mané-Padrós et al., 2008). To assess whether BgHR4 was responsible for coordinating such transcriptional response, we analyzed the expression of these nuclear receptors in prothoracic glands from dsBgHR4-1- and dsMock-treated last instar nymphs at the end of the instar (Fig. 7A). Whereas the expression of *BgHR3*, *BgEcR-A* and *BgRXR* was not affected in BgHR4 knockdown nymphs, the repression of *BgE75A*, *BgE75B* and *BgE75E* was significantly impaired and the activation of *BgFTZ-F1* was totally halted. However, since BgHR3 is also necessary for *BgFTZ-F1* expression (Cruz et al., 2008) and its levels are affected by the absence of BgHR4, we wondered whether the lack of *BgFTZ-F1* expression was a consequence of the high levels of the BgE75 isoforms that, in turn, would impair the activity of *BgHR3*. To test this possibility, we determined the *BgFTZ-F1* mRNA levels on day 6 of the last nymphal instar in dsBgE75-treated nymphs (a treatment that induces the premature expression of *BgHR3* and *BgHR4*), and in nymphs treated simultaneously with dsBgE75 and dsBgHR4-1 (which induces the expression of *BgHR3* but not of *BgHR4*). As Fig. 7B shows, whereas the levels of *BgFTZ-F1* were negligible in dsMock-treated nymphs, those in BgE75 knockdown nymphs were very high, as high as in those treated with dsBgE75 and dsBgHR4-1.

Taken together, these results indicate that the effect of BgHR4 on the activation of *BgFTZ-F1* is not direct but rather it acts through the repression of *BgE75* expression at the end of the instar, thus allowing the remaining BgHR3 to induce *BgFTZ-F1* at this precise moment.

4. Discussion

Using RNAi in vivo in *B. germanica*, we previously demonstrated that 20E, acting through its heterodimer receptor BgEcR-A/BgRXR, directly induces the activation of a stereotypic cascade of the nuclear hormone receptors *BgE75*, *BgHR3* and *BgFTZ-F1*, which controls the developmental progression of the cockroach (Cruz et al., 2006, 2007, 2008; Martín et al., 2006; Mané-Padrós et al., 2008, 2010). In this study, we have isolated and characterized BgHR4 in *B. germanica* as a new member of this hierarchy.

4.1. Expression patterns and hormonal regulation of BgHR4

Expression studies show that *BgHR4* is always present during the decline of the ecdysteroid pulses that occur during the life cycle of *B. germanica*. The expression of *BgHR4* during embryogenesis suggest that it could be involved in the dorsal closure and the deposition of the first cuticle on days 6–7 and in the deposition of a subsequent cuticle layer on days 13–17 (Fig. 2A). Interestingly, *BgHR4* is also detected on day 2, when no ecdysteroid peak is observed. However, we presume that

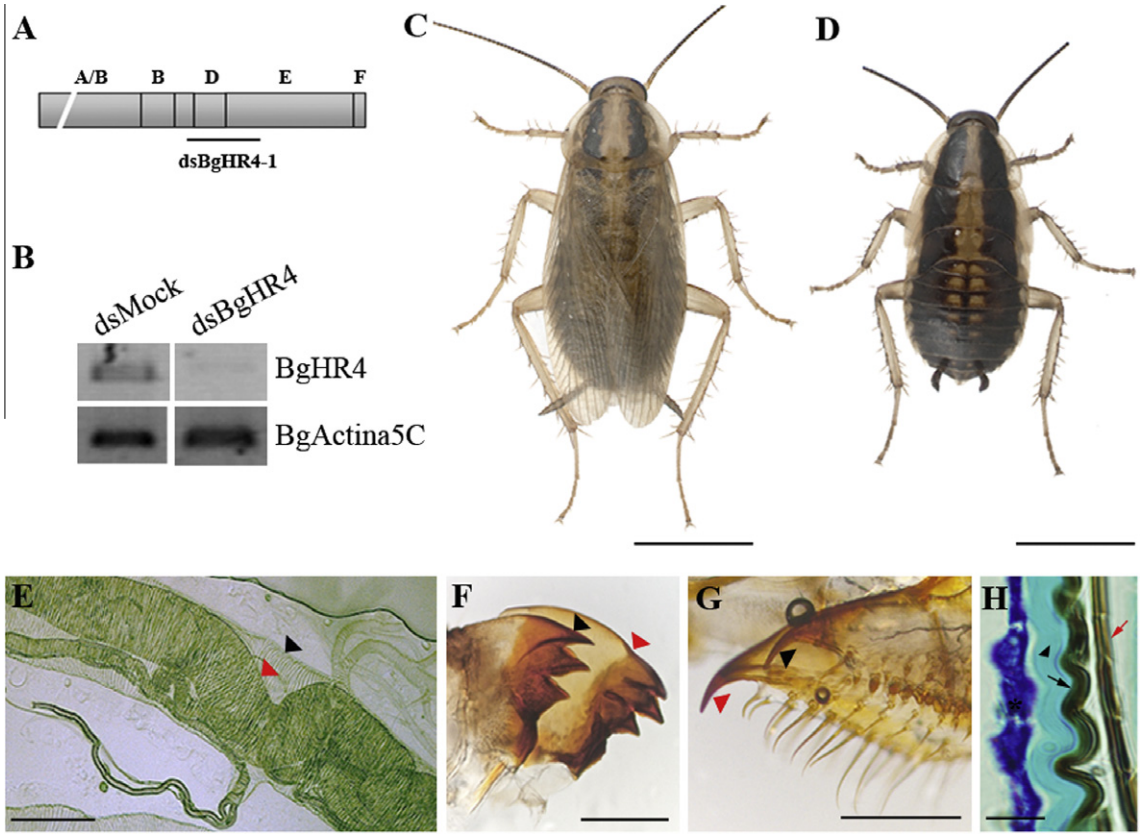

Fig. 5. Silencing of BgHR4 by RNAi in vivo in sixth instar female nymphs of *B. germanica*. (A) Scheme of BgHR4 domain organization showing the region used to generate the dsRNA. (B) One microgram of dsBgHR4-1 was injected in newly emerged sixth instar female nymphs, and mRNA levels were determined 8 days later in the prothoracic gland by RT-PCR/Southern blotting. Equivalent experiments injecting a non-specific sequence (dsMock) served as negative control. *BgActin5C* levels were used as a reference. The Southern blots shown are representative of 10 replicates. (C–H) Effect of RNAi of BgHR4. Newly emerged sixth instar female nymphs were injected with 1 μg of dsBgHR4-1 or with dsMock and left until the time of imaginal molt. (C) dsMock-treated specimen completing the imaginal molt, showing normal winged adult appearance. (D) dsBgHR4-1-treated specimen of the same age arresting development without molting. (E and H) The arrested specimens show duplication of ectodermal-derived structures (nymphal structures indicated with a red arrowhead, and adult structures with black arrowheads), like superimposed trachea (E), double mandibles (F) and double lacinia (G). (H) Cuticle layers of an arrested specimen showing the nymphal epicuticle (red arrow) above the adult endocuticle (black arrowhead) and epicuticle (black arrow) as well as the epidermis (red asterisk). Scale bars: 0.5 mm in C and D; 200 μm in E; 500 μm in F and G; 50 μm in H (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

there may be an ecdysteroid pulse around this period because we have also detected transient expressions of two 20E-dependent genes, *BgE75A* (Mané-Padrós et al., 2008) and *BgHR3* (Martín et al., unpublished). In comparison with *B. germanica*, during the embryogenesis of *D. melanogaster*, the only insect where the embryonic expression of this factor has been reported, *DHR4* is detected for a relatively brief temporal window at 10–14 h after egg laying, coinciding with the decline of the single mid-embryonic ecdysteroid pulse (Sullivan and Thummel, 2003).

In contrast with the differences in *HR4* expression during the embryonic development of *B. germanica* and *D. melanogaster*, the timing of *BgHR4* expression in the prothoracic gland, epidermis and fat body during nymphal development of *B. germanica* is very similar to those described during different post-embryonic stages of the holometabolous insects *T. molitor* (Mouillet et al., 1999), *B. mori* (Charles et al., 1999), *M. sexta* (Weller et al., 2001) and *D. melanogaster* (Sullivan and Thummel, 2003).

The expression pattern of *BgHR4* suggests that 20E is involved in its regulation. Fat body incubations in vitro confirmed that *BgHR4* is activated by 20E and that a putative 20E-inducible inhibitor factor, possibly BgHR4 itself, would be involved in the regulation of *BgHR4* expression. Again, this type of 20E-responsiveness is similar to that described for *HR4* in *M. sexta* and *A. aegypti* (Hiruma and Riddiford, 2001; Cruz et al., 2009). Conversely, in *D. melanogaster*, although *DHR4* is a direct target of 20E, the maximal expression of *DHR4* requires the synergistic activity of a 20E-induced protein (King-Jones et al., 2005; Gauhar et al., 2009).

4.2. Role of BgHR4 in the ecdysteroid-triggered cascade of *B. germanica*

In *B. germanica*, periodic pulses of 20E at the end of each nymphal instar act as developmental timer triggering the molt to the next stage. 20E functions through a complex cascade of nuclear receptors that responds to, and transduces the hormonal signal. The results presented here, clearly demonstrated that BgHR4 contributes decisively to the crossregulatory interactions among these nuclear receptors (Fig. 8).

This complex hierarchy is initiated by the increase of ecdysteroid levels at mid-nymphal instar, which sequentially induces *BgE75C*, *BgE75A* and *BgE75B* as a result of a regulatory crosstalk between them (Mané-Padrós et al., 2008). These BgE75 isoforms, in turn, repress the early activation of *BgHR3* until ecdysteroids reach the highest levels (Fig. 4B) (Mané-Padrós et al., 2010). The specific BgE75 isoform responsible for *BgHR3* repression has not been identified, but probably all three would have repressor properties given that our previous experiments of BgE75 isoform-specific interference have shown that all isoforms have redundant functions (Mané-Padrós et al., 2008). At the peak of the ecdysteroid pulse, BgE75 isoforms cannot longer inhibit *BgHR3* expression. BgHR3 then activates *BgHR4* as the ecdysteroid titer declines

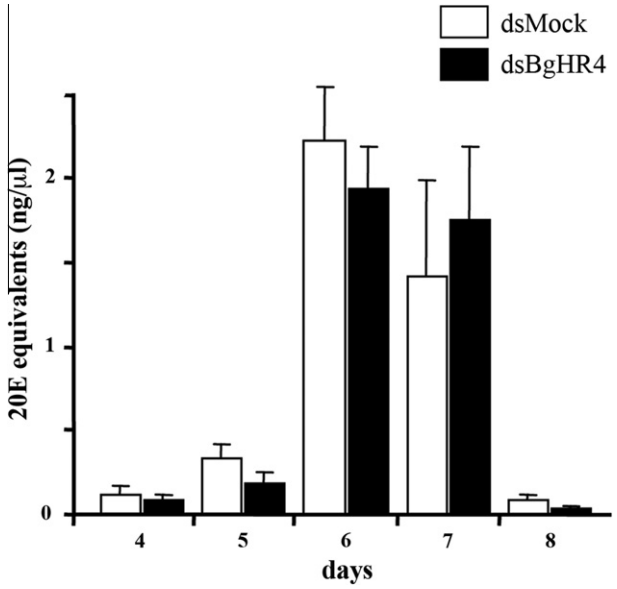

Fig. 6. Effect of RNAi of BgHR4 on ecdysteroid levels in *B. germanica*. Hemolymph from dsBgHR4-1 and dsMock-treated nymphs was collected at the days indicated of the sixth nymphal instar, and ecdysteroids levels were determined by ELISA. Results are expressed as ng/µl of 20-hydroxyecdysone (20E) equivalents. Vertical bars indicate the SEM (n = 6–12).

(Fig. 4A and C). Finally, BgHR4 down-regulates *BgE75A*, *BgE75B* and *BgE75E* (Fig. 7A), allowing BgHR3 to induce *BgFTZ-F1* at the end of the nymphal stage (Fig. 8).

Interestingly, the 20E-triggered cascade described above is also present in more derived holometabolous insects, such as *M. sexta* and *D. melanogaster* (King-Jones et al., 2005; Hiruma and Riddiford, 2009). However, although the architecture of the hierarchy is mostly conserved, the regulatory interplay between E75, HR3, HR4 and FTZ-F1 differs between hemimetabolous and holometabolous species. Although in *D. melanogaster* this cascade is recurrently present following the mid-embryonic, second and third-instar ecdysteroids pulses, it has been mainly characterized during the early stages of metamorphosis. Thus, in the prepupal stage of the fruitfly, as happens in *B. germanica*, DHR3 and DHR4 act together to induce *βFTZ-F1* expression (Lam et al., 1999; King-Jones et al., 2005). However, in contrast to the cockroach, *DHR4* expression in *D. melanogaster* begins before that of *DHR3*, thus suggesting that the *DHR4* activation is independent of DHR3 (Sullivan and Thummel, 2003; King-Jones et al., 2005). In *M. sexta*, GV1 cell transfection assays demonstrated that, as occurs in *B. germanica*, ME75A represses the induction of *MHR3* (Hiruma and Riddiford, 2004). However, two features vary from *B. germanica*. First, MHR3 represses the 20E-dependent induction of *MHR4* (Hiruma and Riddiford, 2007), and second, MHR4 would act as a transcriptional repressor of *MβFTZ-F1* (Riddiford et al., 2003). Furthermore, although there is no functional analysis of AaHR3 and AaHR4 in the mosquito *A. aegypti*, the expression patterns of both receptors suggest that the same regulatory interplay would also occur in the mosquito fat body during adult vitellogenesis (Cruz et al., 2009).

4.3. The function of BgHR4 in molting is channelled through BgFTZ-F1

Using RNAi in vivo, we have shown that BgHR4 is required at the end of each nymphal instar to complete the molting process. BgHR4 knockdown nymphs arrest development with the new adult endo- and exocuticle layers formed and the old nymphal endocuticle totally digested (Fig. 5H). The same molting defects were observed in *B. germanica* nymphs with reduced levels of *BgHR3* and *BgFTZ-F1* (Cruz et al., 2007, 2008), which suggest that BgHR3, BgHR4 and BgFTZ-F1 are mainly required for the last step of molting, namely the ecdysis. During this process, a succession of precise body contractions is triggered and controlled by a number of peptides synthesized and released from the CNS and from highly specialized tracheal cells, called Inka cells (Zitnan and Adams, 2005). Interestingly, the molting impairment observed in *B. germanica* has been also reported in *D. melanogaster* when *βFTZ-F1* levels are reduced in Inka cells by RNAi, as the release of

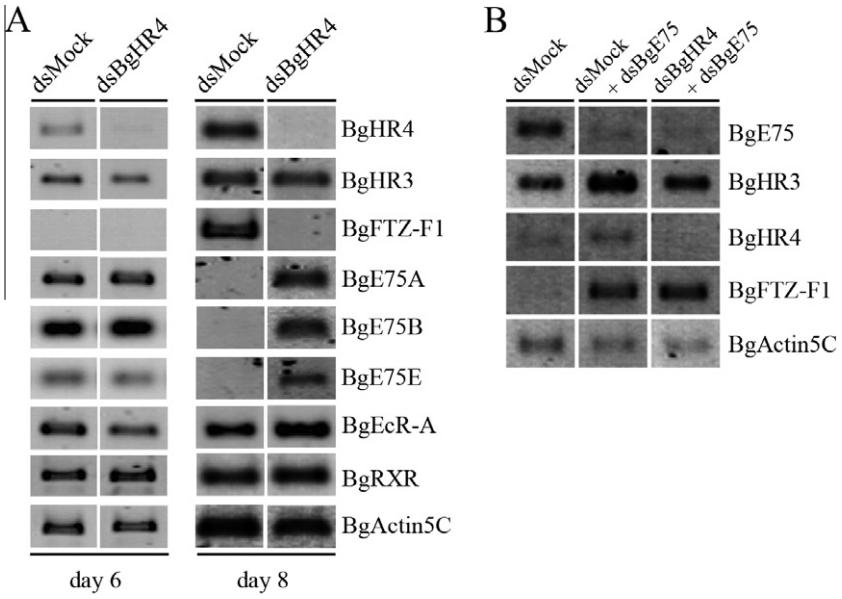

Fig. 7. BgHR4 plays a critical role in the regulation of the ecdysteroid-triggered gene cascade in *B. germanica*. (A) Effect of RNAi of BgHR4 on the expression of other 20-hydroxyecdysone-dependent nuclear receptors. One microgram of dsBgHR4-1 was injected in newly emerged sixth instar female nymphs, and mRNA levels of the selected receptors were determined 6 and 8 days later. (B) Effect of BgHR4 or combined BgHR4 plus BgE75 RNAi on the expression of *BgFTZ-F1*. In all cases, mRNA levels of nuclear receptors were determined by RT-PCR/Southern blotting using the corresponding specific probes. In all cases, *BgActin5C* levels were used as a reference. The Southern blots shown are representative of 10 replicates.

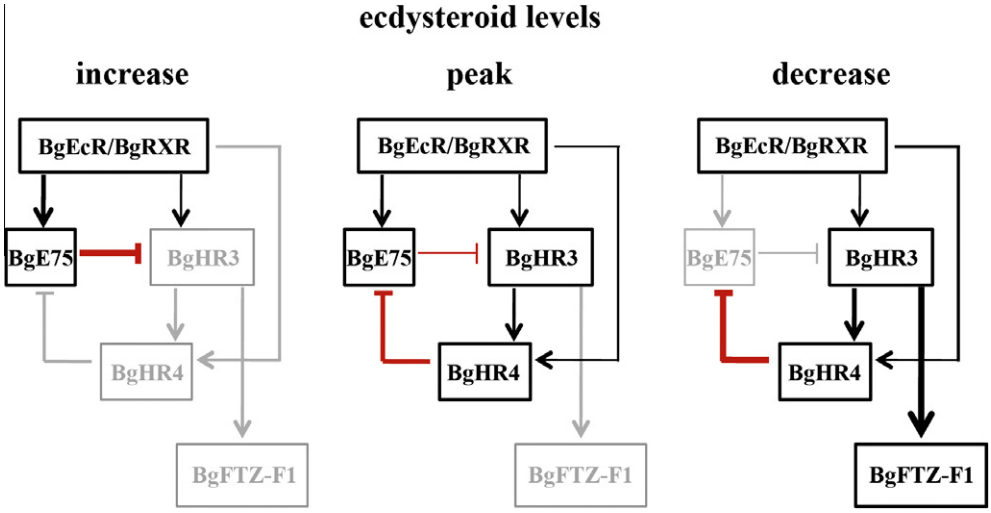

Fig. 8. Representation of the 20-hydroxyecdysone-triggered regulatory interactions during the three sequential steps of the ecdysteroid burst (increase, peak and decrease) occurring in the last nymphal instar of *B. germanica*. Black arrows represent inductive effects and red lines represent repressive effects. Gray colors denote genes and transcriptional regulatory events that are absent during each particular period. The model summarizes the regulatory interactions described in the text and in Cruz et al. (2006, 2007, 2008), Maestro et al. (2005) and Mané-Padrós et al. (2008) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

ecdysis triggering hormone (ETH) from these cells is blocked (Zitnan et al., 2007). Taken together, these results suggest that in *B. germanica*, as in *D. melanogaster*, ecdysis is mainly controlled by BgFTZ-F1 and that the role of BgHR3 and BgHR4 is to provide the necessary delay in *BgFTZ-F1* expression until the end of the instar. It is interesting to note, however, that the synthesis of the new cuticle layers and the digestion of the old endocuticle, although also 20E-dependent in *B. germanica*, are two processes that are independent of BgHR3, BgHR4 or BgFTZ-F1, which suggests the occurrence of other 20E-dependent regulators of the molting cycle yet to be identified.

4.4. Conserved and new functions for the HR4 nuclear receptor

The data presented here is the first functional analysis of the *HR4* gene in a hemimetabolous insect. In holometabolous species, HR4 loss of function analysis has been carried out in the coleopteran *T. castaneum* and in the dipteran *D. melanogaster*. In *T. castaneum*, TcHR4 is required for normal larval-pupal transition and for successful adult reproduction (Tan and Palli, 2008; Xu et al., 2010). In the more derived *D. melanogaster*, disruption of DHR4 function during post-embryonic development results in two different phenotypes: first, *DHR4* last instar mutant larvae display premature wandering behavior and precocious onset of pupariation; and second, *DHR4* mutants arrest development at early stages of metamorphosis mainly due to the absence of a global repression of the ecdysteroid-regulated gene expression (King-Jones et al., 2005). Remarkably, whereas in *B. germanica* BgHR4 function is necessary to complete all the post-embryonic transitions (this study), DHR4 has no essential functions before the pupal stage in the fruitfly, thereby suggesting that this gene has evolved from an ancestral function related with the control of each developmental transition in hemimetabolous insects to a new holometabolous-specific role in coordinating growth and maturation during the last larval stage. In contrast to this specific new role of DHR4, specifically during the prepupal stage of *D. melanogaster* DHR4 has maintained its primitive regulatory function observed in *B. germanica*, acting as a potent transcriptional repressor of several 20E-dependent genes, namely *E75A*, *E75B* and *IMP-L1* among others, and also activating *βFTZ-F1* expression (King-Jones et al., 2005). Interestingly, the repressive function of HR4 has been also observed in *M. sexta* (Hiruma and Riddiford, 2007).

In summary, our work shows that that the nuclear receptor BgHR4 is essential throughout nymphal development in the hemimetabolous insect model *B. germanica*, acting as potent repressor in the 20E-responsive transcriptional cascade that occurs periodically during the post-embryonic development of this cockroach. Furthermore, the comparison of HR4 functions between hemimetabolous and holometabolous species shows that this nuclear receptor may have played a major role in the selection of new-specific regulatory processes associated to the evolution towards holometaboly.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mce.2011.09.025.

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