



## Key roles of the *Broad-Complex* gene in insect embryogenesis

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### ABSTRACT

The *Broad-Complex* (*BR-C*) is a primary response gene in the ecdysteroid signaling pathway which encodes a family of transcription factors that play a key role in metamorphic processes. With the aim of studying the possible functions of *BR-C* in the embryogenesis of a short germ band insect, we cloned *BR-C* cDNAs in the cockroach *Blattella germanica* (*BgBR-C*) and obtained the full coding region of six *BR-C* isoforms (Z1–Z6) of which, Z1–Z5 have homologous sequences in other insects, whereas Z6 is new. Temporal-expression patterns indicate that *BgBR-C* isoforms are present throughout the embryogenesis of *B. germanica*, although with weak fluctuations. Silencing all *BgBR-C* isoforms in the embryo through parental RNAi elicited a diversity of phenotypes. These phenotypes suggest roles for *BgBR-C* in different embryogenesis processes of *B. germanica*, mainly in the formation of the germ band, in the transition from 30% to 35% of total development, when the amnion and serosa rupture and the secondary dorsal organ is formed, and in the hatching process, which involves neural, endocrine and muscular coordination.

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### 1. Introduction

The *Broad-Complex* (*BR-C*) is a primary response gene in the ecdysteroid signaling pathway which encodes a family of C2H2 zinc-finger transcription factors that are involved in a number of insect processes, such as metamorphosis, oogenesis and vitellogenesis (Chen et al., 2004; Deng and Bownes, 1997; Dubrovsky, 2005; Spokony and Restifo, 2007). From a molecular point of view, *BR-C* has been thoroughly studied in the fruitfly *Drosophila melanogaster*, where it encodes four zinc-finger protein isoforms (Z1, Z2, Z3 and Z4) which share a common amino-terminal region called the BRcore, but have a unique pair of zinc-fingers at their carboxy terminus (Bayer et al., 1996; DiBello et al., 1991). Moreover, the common BRcore region contains a BR-C-Tramtrack-Bric-à-brac (BTB) domain that appears to be involved in protein–protein interactions (DiBello et al., 1991). Zinc-finger isoforms of *BR-C* have been described in other insects, and expression studies in the mosquito *Aedes aegypti* (Chen et al., 2004) and in the moths *Manduca sexta* (Zhou et al., 1998) and *Bombyx mori* (Ijiro et al., 2004; Nishita and Takiya, 2004; Reza et al., 2004) confirm that *BR-C* expression is ecdysteroid-dependent.

Metamorphosis is one of the most emblematic processes regulated by *BR-C* (Karim et al., 1993; Zhou et al., 1998). In the

holometabolon *D. melanogaster*, for example, *BR-C* null mutants in which none of the four isoforms is expressed remain in a prolonged larval state and never molt to pupae (Belyaeva et al., 1980; Kiss et al., 1988). Furthermore, the use of a recombinant Sindbis virus expressing a *BR-C* antisense RNA fragment in another holometabolon, namely *B. mori*, was found to reduce endogenous *BR-C* mRNA levels in infected tissues thereby preventing the experimental insects from completing the larval–pupal transition (Uhlirva et al., 2003). Experiments to deplete *BR-C* mRNA levels using systemic RNAi approaches have been carried out in the milkweed bug (hemimetabolon) *Onco-peltus fasciatus* (Erezyilmaz et al., 2006), the flour beetle *Tribolium castaneum* (Konopova and Jindra, 2008; Parthasarathy et al., 2008) and in the lacewing *Chrysopa perla* (Konopova and Jindra, 2008), the latter two of which are basal holometabolon species. The results in *O. fasciatus* suggested that *BR-C* regulates gradual morphogenesis through the successive molts, whereas *BR-C* knockdown in *T. castaneum* and *C. perla* impaired pupal differentiation.

As far as insect embryogenesis is concerned, studies with *D. melanogaster*, a long germ-band species, have shown that *BR-C* is expressed during the second half of embryogenesis (Sullivan and Thummel, 2003) and that the *BR-C* Z3 isoform is found in embryonic neurons (Zhou et al., 2009). However, and as stated above, null mutations that disrupt all *BR-C* isoforms result in developmental arrest and lethality only at pupariation (Belyaeva et al., 1980; Kiss et al., 1988), thus suggesting that *BR-C* is essential for metamorphosis but not for prior developmental events, including

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embryogenesis. In contrast, in the present paper we show that BR-C proteins play a key role in the embryogenesis of the cockroach *Blattella germanica*, a short germ band insect.

## 2. Material and methods

### 2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at  $30 \pm 1$  °C and 60–70% r.h. Newly emerged females were maintained with males during the whole of the first gonadotrophic cycle, and mating was confirmed at the end of experiments by assessing the presence of spermatozooids in the spermatheca. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens. Data on the duration (days) of the reproductive cycle and on the number of nymphs hatched per ootheca are expressed as the mean  $\pm$  SD.

### 2.2. Cloning of BR-C cDNAs

Degenerate primers based on the BTB domain of insect BR-C sequences available in GenBank were used to obtain a *B. germanica* homolog cDNA fragment by RT-PCR. This PCR amplification was carried out using cDNA generated by reverse transcription of poly-A<sup>+</sup> RNA from adult *B. germanica* ovaries as a template. The 360 bp amplified fragment of the BTB domain was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. *B. germanica* isoforms were obtained using a specific primer designed on the BTB domain and degenerate primers designed for each of the Z1–Z4 zinc-finger domains known in *D. melanogaster*. Subsequently, full-length cDNAs were obtained using 5' and 3' rapid amplification of the cDNA ends (5' and 3' RACE). All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and were fully sequenced in both directions. This approach led to isolate the homologs of the *D. melanogaster* Z1–Z4 isoforms and three more cDNAs (Z5, Z6 and Z2/3), which differ in the zinc-finger domain and in the 3'UTR. All primer sequences used in the amplifications are available on request.

### 2.3. Sequence comparisons and phylogenetic analyses

Insect sequences labeled as BR-C isoforms were obtained from GenBank, and the search was enlarged by BLAST using the *B. germanica* BR-C (BgBR-C) sequences as queries. The sequences included in our analysis were those of *Acheta domesticus* (Z1: ABA02190), *Acyrtosiphon pisum* (accession number: XP\_001942681), *A. aegypti* (Z1: AAS80326; Z2: AAS80327; Z3: AAS80328; Z4: AAS80329), *Anopheles gambiae* (XP\_565666; XP\_308027; XP\_308028), *Apis mellifera* (Z1: BAE72138; Z2: BAE72139; Z3: BAE72140; Z4: BAE72137), *B. mori* (Z1: BAD23978; Z2: BAD23979; Z3: BAD24050; Z4: BAD46738), *Culex quinquefasciatus* (EDS39012; XP\_001844997), *D. melanogaster* (Z1: CAA38474; Z2: CAA38476; Z3: CAA38475; Z4: AAB09760), *M. sexta* (Z2: AAC78286; Z3: AAC78287; Z4: AAC78288), *Nasonia vitripennis* (XP\_001602030; XP\_001601347) and *T. castaneum* (Z1: ABW91136; Z2: ABW91137; Z3: ABW91138; Z4: ABW91139; Z5: ABW91135). These zinc-finger domain sequences were aligned with those obtained in *B. germanica* using ClustalX (Thompson et al., 1997) and the resulting alignment was analyzed with the PHYML program (Guindon and Gascuel, 2003), which is based on the maximum-likelihood principle with the amino acid substitution model. Four substitution rate categories with a gamma shape parameter of 1.444 were used and the data were bootstrapped for 100 replicates using the same program.

### 2.4. RT-PCR/Southern blot analyses

RT-PCR followed by Southern blotting with specific probes was used to establish the expression patterns of BgBR-C. To monitor mRNA expression, total RNA was isolated from oothecae with embryos at different developmental stages. All RNA extractions were performed using the GenElute Mammalian Total RNA kit (Sigma, Madrid, Spain). An amount of 400 ng of each RNA extract was treated with DNase (Promega, Madison, WI, USA) then reverse transcribed with Superscript II reverse transcriptase (Invitrogen, Carlsbad CA, USA) and random hexamers (Promega). The quality and amount of RNA were estimated by spectrophotometric absorption at 260 nm in a Nanodrop Spectrophotometer ND-1000H (NanoDrop Technologies, Wilmington, DE, USA). The forward primer was based on the BRcore region, and the reverse primer was based on the zinc-finger domain of each isoform except for Z2 where the reverse primer was designed on the corresponding 3'UTR in order to discriminate it from Z2/3. Finally, a primer pair designed within the BRcore was used to monitor the global expression of all isoforms. cDNA samples were subjected to PCR with a number of cycles within the linear amplification range for each transcript (Ciudad et al., 2006). cDNA probes for Southern blot analyses were generated by PCR using the same primer pairs. The probes were labeled with fluorescein using the Gene Images random prime labeling module (GE Healthcare). RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, thus indicating that there was no genomic contamination. The actin 5c gene of *B. germanica* (Accession number AJ862721) was used as a reference. All primer sequences used in the amplifications are available on request.

### 2.5. RNA interference

A dsRNA encompassing a 623 bp sequence within the BRcore region (Fig. 1), labeled as dsBgBR-C-1, was prepared, then amplified by PCR and cloned into the pSTBlue-1. A non-coding sequence from the pSTBlue-1 vector (dsMock) was used as control dsRNA. The dsRNAs were prepared as described previously (Gomez-Orte and Belles, 2009). Newly emerged adult females were injected with 1 µg of dsBgBR-C-1 in a volume of 1 µl in the abdomen. Control specimens were injected with the same volume and dose of dsMock. An alternative dsRNA (dsBgBR-C-2), which encompasses a 423 bp sequence in the BRcore region (Fig. 1) and was prepared following the same methodology, was used to assess the specificity of the effects obtained with dsBgBR-C-1.

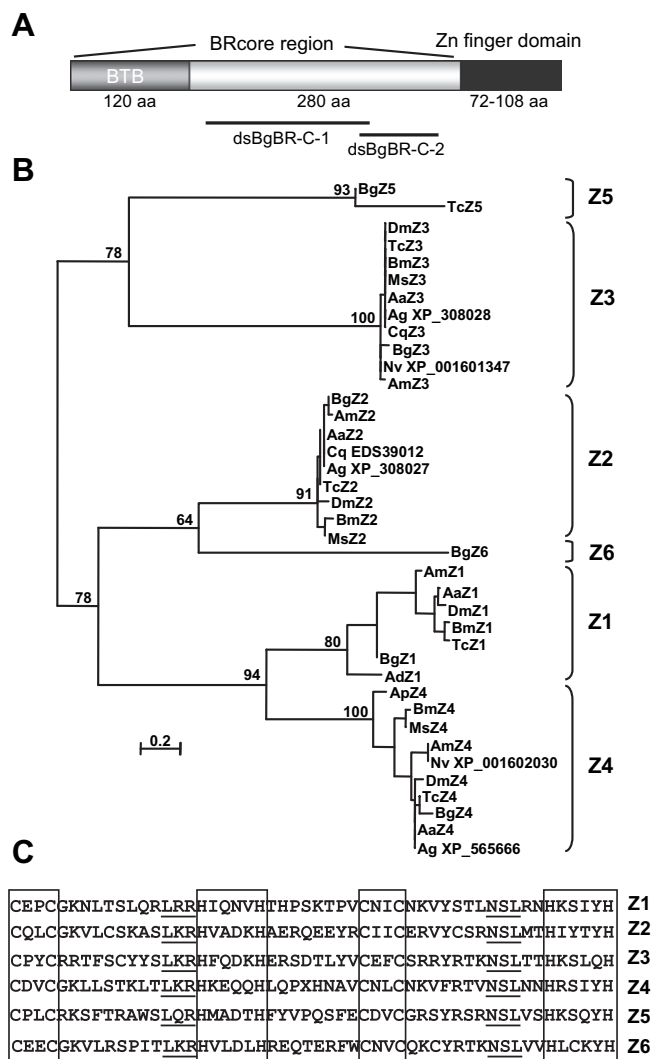
### 2.6. Microscopy

Oothecae of different ages were detached from the female abdomen by gentle pressure. After 5 min in a water bath at 95 °C, each ootheca was opened and the embryos dechorionated and individualized. Between 6 and 12 embryos per ootheca, chosen from the central part, were dissected for staining and examination. These embryos were fixed in 4% paraformaldehyde in PBS for 2 h, then washed with PBS 0.3% Triton (PBT) and incubated for 5 min in 1 mg/ml DAPI in PBT. They were then mounted in Mowiol (Calbiochem, Madison, WI, USA) and observed with a Zeiss Axiophot microscope (Carl Zeiss MicroImaging).

## 3. Results

### 3.1. BR-C cDNAs of *B. germanica*

In a first step, a fragment of BR-C cDNA was cloned by RT-PCR using adult ovarian tissue from *B. germanica* as template and degenerate primers based on conserved motifs of the BTB domain



**Fig. 1.** Broad-complex in *Blattella germanica*. **A:** Protein organization indicating the BTB domain, BRcore and zinc-finger domains. The regions encompassed by the two dsRNAs used in RNAi studies (dsBgBR-C-1 and dsBgBR-C-2) are also indicated. **B:** Phylogenetic analysis of insect BR-C zinc-fingers for *Acheta domesticus* (Ad), *Acyrtosiphon pisum* (Ap), *Aedes aegypti* (Aa), *Anopheles gambiae* (Ag), *Apis mellifera* (Am), *Blattella germanica* (Bg), *Bombyx mori* (Bm), *Culex quinquefasciatus* (Cq), *Drosophila melanogaster* (Dm), *Manduca sexta* (Ms), *Nasonia vitripennis* (Nv) and *Tribolium castaneum* (Tc). The GenBank accession number is provided where the zinc-finger isoform has not been labeled. Bootstrap values are shown for the main basal nodes (those of the more distal nodes are always higher than 50). The scale bar indicates substitutions per position. **C:** Sequence of the six zinc-finger variants, defining the six protein isoforms of *B. germanica* BgBR-C. The characteristic motifs CXXC and HXXXXH are boxed and conserved motifs are underlined.

of BR-C sequences available in GenBank. Subsequent amplification of specific isoforms based on the Z1–Z4 sequences of *D. melanogaster*, followed by 5' and 3' RACE, gave seven cDNAs of 1652, 1722, 1754, 1783, 1862, 1949 and 2041 kb, respectively, rather than the expected four. Conceptual translation of these cDNAs indicated that the corresponding proteins showed the typical BR-C organization, comprising a BTB domain (120 amino acids in length) that presents between 92% and 98% identity with the BTB domain of other BR-C sequences, the remaining portion of the BRcore region (with 280 amino acids), which is quite divergent among species (the closest to *B. germanica* is that of *A. domesticus*, with 47% identity), and the zinc-finger domain, which is quite variable in structure and length (72–80 amino acids), depending on the isoforms (Fig. 1A).

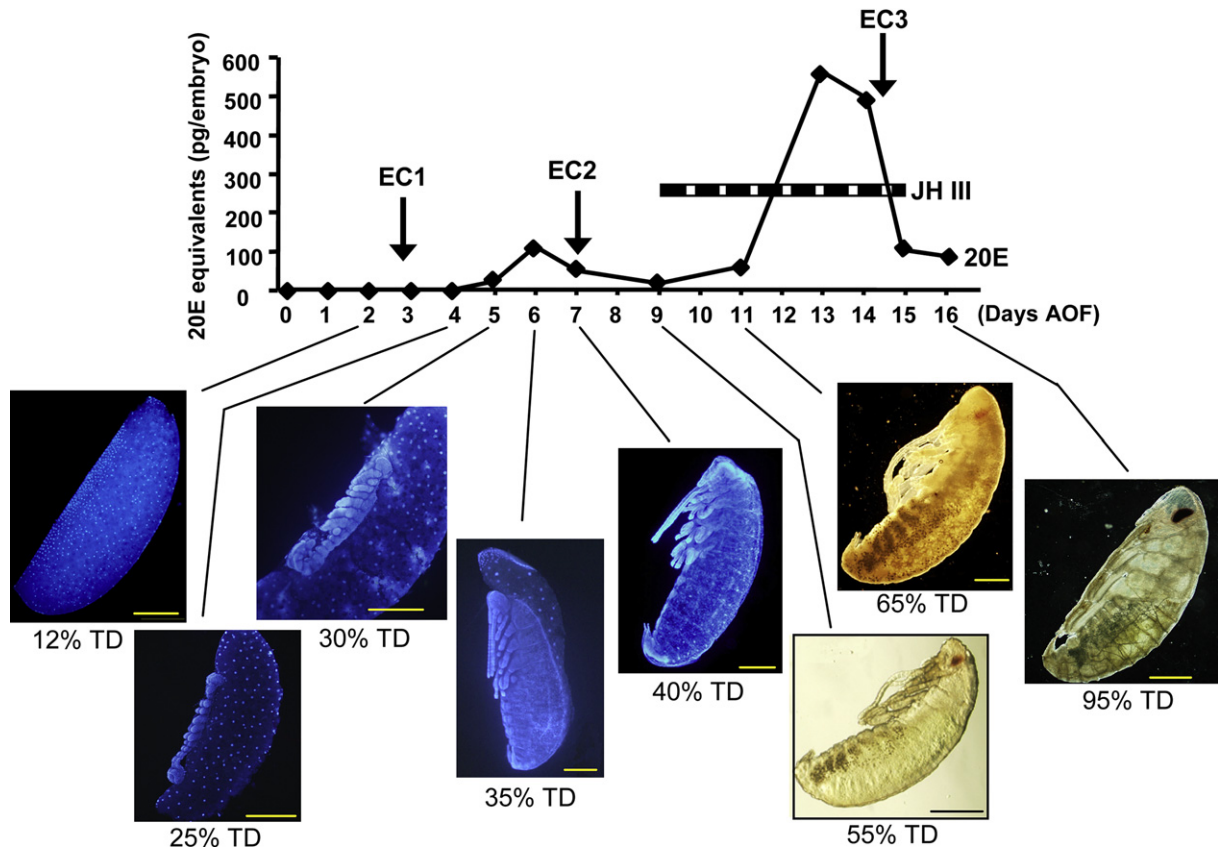
A preliminary alignment of *B. germanica* sequences with known insect BR-C sequences allowed us to readily identify some of the classical zinc-finger based BR-C isoforms known in other insects, such as *D. melanogaster* Z1–Z4 (Bayer et al., 1996; DiBello et al., 1991). Subsequent phylogenetic analysis, following a maximum-likelihood approach using the zinc-finger domain of known BR-C sequences, allowed a coherent nomenclature for the zinc-finger isoforms to be assigned. The results of this analysis (Fig. 1B) indicated that four of the *B. germanica* isoforms appear in the respective nodes of Z1, Z2, Z3 and Z4, and that another one clusters with the recently described *T. castaneum* Z5 (Konopova and Jindra, 2008). A sixth *B. germanica* transcript variant, which we named Z6, appears as the sister group of the Z2 node. Thus, the *B. germanica* isoforms were named BgBR-CZ1 (accession number: FN651774), BgBR-CZ2 (accession number: FN651775), BgBR-CZ3 (accession number: FN651776), BgBR-CZ4 (accession number: FN651777), BgBR-CZ5 (accession number: FN651778) and BgBR-CZ6 (accession number: FN651779). The seventh cDNA found is similar to the Z2 isoform but with a 3'UTR region containing the complete zinc-finger domain sequence of the Z3 isoform. We named this transcript BgBR-CZ2/3 (accession number: FN651780), although its conceptual translation would give the Z2 protein. In summary, a total of six isoform proteins are predicted to be derived from the BgBR-C gene. Fig. 1C, which depicts the zinc-finger domain sequence of these isoforms, shows that they all fit the characteristic organization C-X(2)-C-X(12)-H-X(4)-H-X(8)-C-X(2)-C-X(12)-H-X(4)-H. Those with homologous sequences in other insects (Z1–Z5) have a high degree of identity (73–98%) with them.

### 3.2. Embryogenesis in *B. germanica* and the hormones involved

*B. germanica* is a short germ-band species whose embryogenesis has been described by Anderson (1972), Tanaka (1976) and Konopova and Zrzavy (2005), amongst others. Briefly, eggs are encapsulated into an ootheca and are in metaphase of the first maturation division when freshly oviposited. New maturation divisions occur after sperm penetration, and in the space of a few hours the zygote enters into a cleavage sequence which produces cleavage energids that finally concentrate in the ventral surface of the egg to form the germ band. This process is completed on day 2 after egg laying and ootheca formation (AOF) and represents 13% total development (TD) (Fig. 2). By day 4 AOF (25% TD) all segments have formed, the caudal end has folded and the appendages are recognizable, and by day 5 (30% TD) the appendages have grown posteriorly and the pleuropodia appear in the first abdominal segment, although the amnion and serosa still envelop the embryo. By day 6 (35% TD) the appendages have extended notably and segmentation is clearly apparent; the amnion and serosa have ruptured and the dorsal organ appears. By day 7 (40% TD) the dorsal closure of the body wall is complete and the dorsal organ disappears and becomes enclosed within the wall. Pigmentation of the eyes begins on day 9 (55% TD), and the tips of the antennae and the hind legs reach the third abdominal segment. This pigmentation becomes more apparent by day 11 (65% TD), and the tips of antennae and the hind legs reach the fifth abdominal segment. By day 16 (95–100% TD) the bristles on the cuticle have developed, the tips of the antennae and the hind legs practically reach the last abdominal segment and the pigmentation of eyes and mandibles becomes very conspicuous (Fig. 2).

ELISA-measured ecdysteroids (Maestro et al., 2005) indicate that there is a small peak on day 6 AOF (35% TD) and a much higher peak on day 13 (81% TD; Fig. 2). Ultrastructural studies (Konopova and Zrzavy, 2005) have shown that *B. germanica* embryos deposit three sequential cuticles, known as EC1, EC2 and EC3. Morphological data indicate that EC2 and EC3 deposition, respectively, follows the





**Fig. 2.** Embryogenesis and ecdysteroid and juvenile hormone occurrence in *Blattella germanica*. Ecdysteroid levels, expressed as 20-hydroxyecdysone (20E) equivalents, are taken from Maestros et al. (2005). Data for the period where juvenile hormone III (JH III) is present are taken from J.-L. Maestros, K. Treiblmayr, N. Pascual and X. Belles (unpublished). The arrows indicate deposition of the three successive embryonic cuticles (EC). AOF: After egg laying and ootheca formation. The insets show characteristic stages of embryo development. Stages beyond EC2 cannot be stained by DAPI. TD: total development. Scale bars: 500  $\mu$ m.

two ecdysteroid peaks described above (Fig. 2). Given that EC1 is produced between completion of the germ band and the beginning of appendage development, in other words around day 3 AOF (20% TD), we can assume that a further ecdysteroid pulse, which is not detectable by ELISA measurements, occurs around day 2. Interestingly, our previous data indicate that two-day-old *B. germanica* embryos show a transient but clear expression of the early ecdysteroid-induced gene E75A (Mane-Padros et al., 2008), which supports the hypothesis of a cryptic ecdysteroid pulse on that day. As far as juvenile hormone (JH) is concerned, our data (J.-L. Maestros, K. Treiblmayr, N. Pascual and X. Belles, unpublished) indicate that JH III is present at concentrations of around 1.5 pmol/embryo from day 9 (63% TD) to day 14 (88% TD) (Fig. 2).

### 3.3. Temporal patterns of BgBR-C expression during embryogenesis

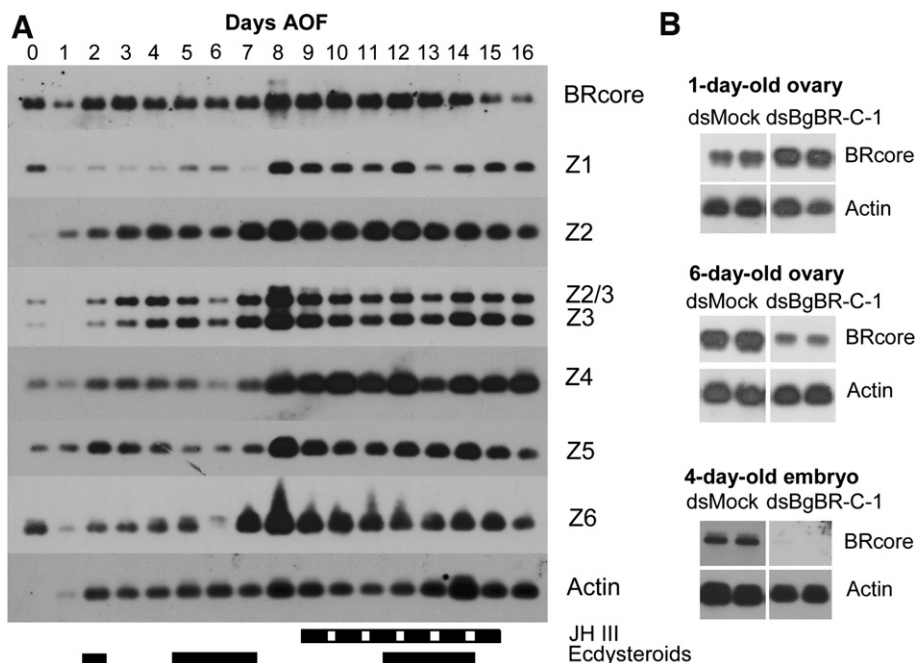
Total RNA was extracted from three independent collections of embryos staged at 24 h intervals throughout the 16 days that *B. germanica* embryogenesis lasts under our rearing conditions. RT-PCR followed by Southern blotting with specific probes designed on the basis of the zinc-finger domain was used to establish the expression pattern of each of the six BgBR-C isoforms (blots Z1–Z6). The joint expression of all these isoforms was studied using primers designed on the basis of the core region (BRcore blot). The procedure was carried out for the three embryo collections, and a blots set representative of the three replicates is shown in Fig. 3A. The double band observed in the Z3 pattern is due to the simultaneous amplification of the Z3 and Z2/3 transcripts as we used a primer pair specific for the Z3 zinc-finger sequence that is

common to both. The heavier band corresponds to the Z2/3 transcript.

The patterns (Fig. 3A) show that, in terms of whole expression (BRcore blot), BgBR-C is present throughout embryogenesis, including in freshly oviposited eggs (0 days). Although the fluctuations are weak, the BRcore pattern suggests the occurrence of two subtle and overlapping expression waves, one after day 2 AOF and another one after day 6. These two possible waves are also apparent in the patterns of individual isoforms, particularly Z2–Z6, whereas the first wave is practically absent from the pattern of Z1. The first wave starts after the possible ecdysteroid pulse that we postulated on day 2 AOF, whereas the second one begins after the small ecdysteroid peak occurring on day 6 (Fig. 2). BgBR-C expression seems to decay after the major ecdysteroid peak centered on day 13 AOF, which coincides with the presence of JH III.

### 3.4. Effects of RNAi on embryo viability and on BgBR-C transcript levels

Parental RNAi was used to silence the expression of all BgBR-C isoforms in *B. germanica* embryos. Thus, a dsRNA encompassing a 623 bp region found within BRcore (dsBgBR-C-1; Fig. 1) was prepared and injected at a dose of 1  $\mu$ g into freshly emerged adult *B. germanica* females. A non-coding sequence (dsMock) injected at the same dose was used as control dsRNA. The duration of the first reproductive cycle and embryo viability was determined initially. Thus, the duration of the first reproductive cycle in dsBgBR-C-1-treated females ( $n = 27$ ) was  $8.51 \pm 1.50$  days, and all of them formed an ootheca, although only  $1.00 \pm 4.03$  nymphs hatched per



**Fig. 3.** Expression of BgBR-C during *Blattella germanica* embryogenesis and effects of RNAi. A: Expression pattern of the six isoforms (Z1–Z6) and whole expression obtained by amplifying a fragment of the core region (BRcore). The presence of ecdysteroids and juvenile hormone III (JH III) is indicated at the bottom (data as in Fig. 2). AOF: After egg laying and ootheca formation. B: Effects of dsBqBR-C-1 on BgBR-C mRNA levels. Newly emerged adult females were treated with 1  $\mu$ g of dsBqBR-C-1 and transcript levels in the ovaries were studied 1 and 7 days later and in the four-day-old embryos. Parallel experiments carried out with dsMock are also indicated. All the blots shown are representative of three replicates.

ootheca. The respective values for dsMock-treated females ( $n = 21$ ) were  $8.33 \pm 0.86$  days and  $38.33 \pm 8.99$  nymphs hatched per ootheca (the normal range is between 35 and 40 nymphs hatched per ootheca). Embryo hatching therefore clearly differs between the two groups, with the oothecae of dsBqBR-C-1-treated females being essentially inviable. The duration of the ootheca transport period was quite constant in dsMock-treated females (between 15 and 17 days) and highly variable in those treated with dsBqBR-C-1 (between 14 and 24 days), with the ootheca being spontaneously dropped without nymph hatching in most of the latter cases.

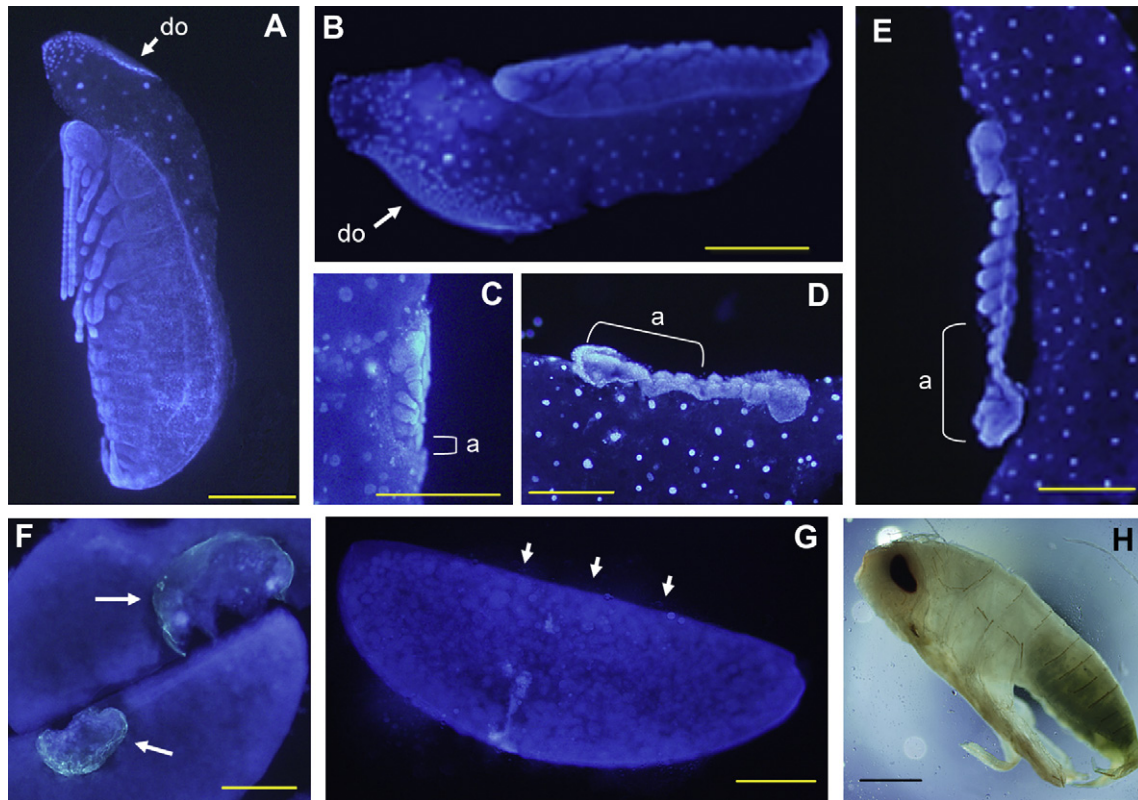
Given that we were expecting a parental RNAi, the same treatment was used to assess the effect on BgBR-C mRNA levels in ovarian tissue taken from the experimental adult females 1 and 6 days after dsRNA application and in the embryos of the first ootheca of these females four days AOF. The results (Fig. 3B) showed that BgBR-C mRNA levels had decreased notably in the ovaries of six-day-old dsBqBR-C-1-treated females and were practically non-existent in four-day-old embryos (Fig. 3B).

The above experiments were repeated using an alternative dsRNA for BgBR-C, this time targeting 423 bp of a different region within the BRcore, which we called dsBqBR-C-2 (Fig. 1A). Using the same dose and approach, the results were virtually identical to those obtained with dsBqBR-C-1. Thus, the duration of the first reproductive cycle in dsBqBR-C-2-treated females ( $n = 18$ ) was  $8.44 \pm 1.72$  days, and only  $1.55 \pm 6.60$  nymphs hatched per ootheca. The respective values for females treated in parallel with dsMock ( $n = 14$ ) were  $8.78 \pm 1.48$  days and  $39.86 \pm 5.26$  nymphs hatched per ootheca. The duration of the ootheca transport period was also very similar, with considerable delays being observed in dsBqBR-C-2-treated females. The effect of dsBqBR-C-2 treatment on BgBR-C mRNA levels showed similar transcript depletions in the ovaries of six-day-old females and four-day-old embryos to those observed with dsBqBR-C-1 (not shown).

### 3.5. Effects of BgBR-C RNAi on *B. germanica* embryogenesis

The same parental RNAi protocol, that is injecting 1  $\mu$ g dsBqBR-C-1 into freshly emerged adult females as well as dsMock at the same dose and day, was used to study the effects of depleting all BgBR-C isoforms on embryo development. In a first set of experiments, the oothecae of treated females were dissected on day 6 AOF (35% TD). All embryos from dsMock-treated females (52 oothecae and 416 embryos examined) showed the normal morphology at this stage (Fig. 4A), whereas the embryos from dsBqBR-C-1-treated females (302 oothecae and 1812 embryos examined) showed a range of phenotypic defects which were classified into five categories as follows. Category 0 is defined by morphologically normal embryos identical to those of the controls (Fig. 4A). Category 1 is represented by embryos morphologically similar to the controls but with a smaller abdomen, often narrower dorso-ventrally (Fig. 4B). Category 2 is represented by embryos arrested at 25–30% TD, with the head, thorax and abdomen differentiated but poorly developed, most of which show ill-defined or practically absent abdominal segments; they cannot rupture the amnion and serosa and do not form the dorsal organ (Fig. 4C–E). Category 3 is defined by the presence of an amorphous mass of distinct tissue in the ventral part of the egg, but with no traces of segmentation (Fig. 4F). Finally, category 4 is represented by embryos that do not form the germ band and show only a very low concentration of energids in the ventral part of the egg (Fig. 4G). The percentages of occurrence of each one of these five categories are summarized in Table 1. It is worth mentioning at this point that a single ootheca can present up to three different malformation categories.

To assess the specificity of the effect of RNAi on embryo development, the above experiments were repeated using the alternative dsBqBR-C-2 (Fig. 1A) at the same treatment dose and age. Oothecae were dissected at day 6 AOF. In this set of experiments, all embryos from the oothecae of dsMock-treated females (32



**Fig. 4.** Effects of parental BgBR-C RNAi on embryogenesis of *Blattella germanica* at day 6 and day 20 AOF. A: Control embryo at day 6 (35% TD). B: Category 1 phenotype at day 6 (embryos morphologically similar to controls, but with a smaller abdomen). C–E: Category 2 at days 6 or 20 (embryo arrested at 25–30% TD, head, thorax and abdomen differentiated but poorly developed). F: Category 3 at days 6 or 20 (only an amorphous mass of distinct tissue is visible in the ventral part of the egg, with no traces of segmentation). G: Category 4 at days 6 or 20 (embryos that did not form the germ band). H: Category 1/20 at day 20 (embryos practically fully developed but with a smaller abdomen; they are unable to hatch). do: dorsal organ; a: region corresponding to the abdomen in malformed embryos. The arrows in F indicate the amorphous mass of distinct tissue and those in G the ventral part of the egg where the germ band should be formed. Scale bars: 500 µm.

oothecae and 256 embryos examined) showed a normal morphology at 35% TD (Fig. 4A). As expected, the eggs taken from the oothecae of dsBgBR-C-2-treated females (91 oothecae and 819 embryos examined) contained embryos showing the characteristic malformation categories observed in the experiments carried out with dsBgBR-C-1, with similar percentages of occurrence for each category (Table 1).

At this point, we wondered whether the phenotypes observed might simply be the consequence of a slower development, therefore we carried out a second set of experiments following the same RNAi protocol but dissecting the oothecae at day 20 AOF, that is four days after the usual hatching day in controls. All oothecae from dsMock-treated females (22 oothecae and 176 embryos examined) hatched between days 16 and 17 AOF and gave normal nymphs, whereas none of the oothecae from dsBgBR-

C-1-treated females (88 oothecae and 616 embryos examined) gave hatched nymphs at day 20. The embryos from these 88 inviable oothecae showed category 2, 3 and 4 malformations, with percentages of occurrence similar to the previous experiments (Table 1). They also showed exactly the same phenotype as in the former experiments, in other words they did not progress beyond the morphological state observed when the oothecae were dissected on day 6 AOF. In addition, 46.75% of the embryos reached practically complete development, including deposition of the third embryonic cuticle, but they did not hatch and had a smaller than normal abdomen (Fig. 4H). We presume that this phenotype (category 1/20, Table 1), corresponds to the category 1 phenotype observed on day 6 AOF (embryos morphologically similar to controls, but with a smaller abdomen narrowed dorso-ventrally).

**Table 1**

Summary of the RNAi experiments carried out to deplete BgBR-C in embryos of *Blattella germanica*, and results in terms of distribution of embryos into phenotype categories 0 to 4. AOF: After egg laying and ootheca formation.

dsRNA	Day of dissection AOF	Oothecae studied	Embryos examined	Category 0	Category 1	Category 1/20	Category 2	Category 3	Category 4
dsBgBR-C-1	6	302	1812	11 (0.6%)	827 (45.64%)	—	203 (11.20%)	377 (20.81%)	394 (21.74%)
dsMock	6	52	416	416 (100%)	0 (0%)	—	0 (0%)	0 (0%)	0 (0%)
dsBgBR-C-2	6	91	819	0 (0%)	357 (43.59%)	—	66 (8.06%)	173 (21.12%)	223 (27.23%)
dsMock	6	32	256	256 (100%)	0 (0%)	—	0 (0%)	0 (0%)	0 (0%)
dsBgBR-C-1	20	88	616	0 (0%)	—	288 (46.75%)	24 (3.90%)	127 (20.62%)	177 (28.73%)
dsMock	20	22	176	176 (100%)	—	0 (0%)	0 (0%)	0 (0%)	0 (0%)



## 4. Discussion

### 4.1. *B. germanica* expresses six protein BR-C isoforms

We have reported the cloning and characterization of six isoforms of BR-C in *B. germanica* which are characterized by a distinctive zinc-finger domain. The fact that the nucleotide sequence in all other domains is identical in the six isoforms suggests that they are splice variants derived from the same gene, as occurs in other insects such as *D. melanogaster* (Bayer et al., 1996; DiBello et al., 1991), *B. mori* (Ijiro et al., 2004; Nishita and Takiya, 2004) and *T. castaneum* (Konopova and Jindra, 2008).

The classification and nomenclature of the isoforms of *B. germanica* was based on a phylogenetic analysis of the zinc-finger domains of insect BR-C isoforms available in GenBank. Of these isoforms, Z1–Z4, which have been thoroughly described in *D. melanogaster* (Bayer et al., 1996; DiBello et al., 1991), are the most common among insects (Spokony and Restifo, 2007) and are also present in *B. germanica* (Fig. 1B). Moreover, our study allowed to ascertain that the two unlabeled BR-C isoforms identified in *N. vitripennis* (accession numbers XP\_001601347 and XP\_001602030) correspond to Z3 and Z4, respectively, that of *C. quinquefasciatus* (accession number EDS39012) corresponds to Z2, that of *A. pisum* (accession number: XP\_001942681) correspond to Z4, and those of *A. gambiae* (accession numbers XP\_308027, XP\_308028 and XP\_565666) correspond to Z2, Z3 and Z4, respectively (Fig. 1B). Z5 was recently discovered in *T. castaneum* (Konopova and Jindra, 2008) and also occurs in *B. germanica* (Fig. 1B). The new isoform Z6 discovered in *B. germanica* is likely to derive from the last ancestor common to the Z2 group. Z6 perhaps arose in cockroaches through Z2 duplication after the splitting of holometabolans, or is ancestral and has been lost in holometabolans. Although complete information on BR-C isoforms is restricted to a few species, the data set suggests that more-derived species have fewer zinc-finger domain isoforms than less-derived ones.

### 4.2. BgBR-C is expressed throughout embryogenesis

Three cuticle depositions take place during *B. germanica* embryogenesis (Anderson, 1972; Konopova and Zrzavy, 2005; Tanaka, 1976). The first of these occurs towards day 3 AOF and is probably preceded by an ecdysteroid pulse, although this could not be confirmed by our ELISA measurements (Maestro et al., 2005). We suppose that there is an ecdysteroid pulse at this stage because two-day-old *B. germanica* embryos show a transient expression of the ecdysteroid-dependent gene *E75A* (Mane-Padros et al., 2008). In addition, new results (J. Cruz, X. Bellés and D. Martín, unpublished) indicate that *HR3*, another early-late gene of the ecdysteroid-induced cascade, also shows an expression pulse at day 2 of *B. germanica* embryogenesis. Our experiments have shown that expression of both *HR3* and *E75A* in *B. germanica* is rapidly induced by 20-hydroxyecdysone (Cruz et al., 2007; Mane-Padros et al., 2008). The second and third cuticle depositions that occur during *B. germanica* embryogenesis take place on days 7 and 14 AOF, respectively, and are preceded by clear ecdysteroid peaks on days 6 and 13 AOF (Maestro et al., 2005). The latter is a peak which overlaps with a large juvenile hormone III peak (J.-L. Maestro, K. Treiblmayr, N. Pascual and X. Belles, unpublished data).

Expression studies indicate that BgBR-C isoforms are more or less expressed throughout embryogenesis in *B. germanica* (Fig. 3A), including in freshly oviposited eggs (0 days), thus pointing to a maternal origin for these transcripts. Two possible expression waves seem to follow the cryptic ecdysteroid pulse on day 2 AOF (12% TD) and the small peak on day 6 AOF (50% TD), respectively, thereby suggesting that BgBR-C expression might be

induced by ecdysteroids in these two stages, although the response occurs later than usual for this early gene of the ecdysteroid-elicited cascade. However, this situation is similar to that described for *D. melanogaster* embryos, where BR-C is expressed just after, but not simultaneously with, the ecdysteroid peak occurring between 25 and 50% TD (Sullivan and Thummel, 2003). Moreover, the pattern observed for *D. melanogaster*, where BR-C is expressed only in the second half of embryogenesis, contrasts with that of *B. germanica*, where BgBR-C mRNA is present during practically the entire process. The use of a primer pair specific for the Z3 zinc-finger sequence, which is common to transcripts Z3 and Z2/3, allowed us to study the pattern of both mRNA species simultaneously, thus indicating that both transcripts are expressed in a parallel fashion. A homologous Z2/3 cDNA has been amplified in *B. mori*, although the Z3 transcript, which was predicted from the genome data, has not been isolated empirically (Ijiro et al., 2004; Nishita and Takiya, 2004).

### 4.3. BgBR-C has key roles in embryo development

Expression of BR-C throughout the second half of *D. melanogaster* embryo development (Sullivan and Thummel, 2003) would suggest a possible function in the embryo. Nevertheless, null mutations disrupting all BR-C isoforms result in lethality only at the onset of metamorphosis (Belyaeva et al., 1980; Kiss et al., 1988), and there is no evidence that BR-C plays a role in embryogenesis. Moreover, it appears that there is no maternal effect of BR-C on embryo development (Perrimon et al., 1989). In contrast, our results show that depletion of mRNA levels of all BgBR-C isoforms in *B. germanica* embryos via parental RNAi elicits a range of defects during embryogenesis. Thus, around half of all embryos do not form the germ band (Category 4) or form a condensate but amorphous germ band (Category 3). Furthermore, around 10% form the germ band but do not progress beyond 25–30% TD (Category 2); in these cases, the amnion and serosa still envelop the embryo, which is often smaller, especially the abdomen. Finally, approximately 40% of embryos practically complete embryogenesis, but cannot hatch and often show a smaller abdomen (Categories 1 and 1/20). This differential penetrance of each phenotype allowed to unveil the role played by BgBR-C at different levels, specifically 1) in the formation of the germ band, 2) in the progress beyond 25–30% TD, when the amnion and serosa rupture and the secondary dorsal organ is formed, and 3) in the hatching process, which involves neural, endocrine and muscular coordination.

The observation of stage- and tissue-specific BR-C isoforms in *D. melanogaster* suggests that they may have distinct functions (Emery et al., 1994; Spokony and Restifo, 2007). Indeed, specific functions for particular isoforms have been elucidated through functional studies in *D. melanogaster*, for example in flight muscles (Sandstrom et al., 1997) and in other tissues and processes (see Spokony and Restifo (2007), and references therein), as well as in *M. sexta* (Zhou and Riddiford, 2001, 2002) and in *B. mori* (Uhlirva et al., 2003; Wang et al., 2009). There are even cases where the expression pattern suggests that different isoforms are functionally redundant but detailed studies have shown that they are actually acting at different times and in different cell types (Spokony and Restifo, 2007, 2009). The structure and temporal-expression patterns of the six isoforms of BgBR-C in *B. germanica* embryos suggest that they might have specific roles. For example, the poor or practically inexistent expression of Z1 from day 1 (5% TD) to day 5 (30% TD) suggests that this isoform does not contribute to the category 2 phenotype. Unveiling possible specific functions of individual BgBR-C isoforms would require further studies involving selective RNAi of each isoform and careful identification of the differential phenotypes.

Finally, a paper describing the role of the Z1 isoform of BR-C in the embryogenesis of the heteropteran *O. fasciatus*, an intermediate germ-band species, appeared while our manuscript was under review (published online 30 January 2010) (Erezylmaz et al., 2009). The results described therein show that this isoform (OfBR-CZ1) is ubiquitously expressed during germ-band invagination and segmentation of *O. fasciatus*, and that RNAi of this isoform elicits malformations ranging from posterior truncations to complete fragmentation of embryo tissues. In light of all the available data, it appears that BR-C transcription factors are conspicuously expressed during important stages of embryogenesis of short and intermediate germ-band insect species (*B. germanica*, *O. fasciatus*), where they play key roles in embryo development. This contrasts with the situation in long germ-band species (*D. melanogaster*), where BR-C transcription factors are less prominently expressed during embryogenesis and appear to have no clear role in this process.

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