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# Broad-Complex acts downstream of Met in juvenile hormone signaling to coordinate primitive holometabolan metamorphosis

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Metamorphosis of holometabolous insects, an elaborate change of form between larval, pupal and adult stages, offers an ideal system to study the regulation of morphogenetic processes by hormonal signals. Metamorphosis involves growth and differentiation, tissue remodeling and death, all of which are orchestrated by the morphogenesis-promoting ecdysteroids and the antagonistically acting juvenile hormone (JH), whose presence precludes the metamorphic changes. How target tissues interpret this combinatorial effect of the two hormonal cues is poorly understood, mainly because JH does not prevent larval-pupal transformation in the derived *Drosophila* model, and because the JH receptor is unknown. We have recently used the red flour beetle *Tribolium castaneum* to show that JH controls entry to metamorphosis via its putative receptor Methoprene-tolerant (Met). Here, we demonstrate that Met mediates JH effects on the expression of the ecdysteroid-response gene *Broad-Complex* (*BR-C*). Using RNAi and a classical mutant, we show that *Tribolium BR-C* is necessary for differentiation of pupal characters. Furthermore, heterochronic combinations of retarded and accelerated phenotypes caused by impaired *BR-C* function suggest that besides specifying the pupal fate, *BR-C* operates as a temporal coordinator of hormonally regulated morphogenetic events across epidermal tissues. Similar results were also obtained when using the lacewing *Chrysopa perla* (Neuroptera), a member of another holometabolous group with a primitive type of metamorphosis. The tissue coordination role of BR-C may therefore be a part of the Holometabola groundplan.

**KEY WORDS: Metamorphosis, Juvenile hormone, Broad-Complex, Methoprene-tolerant, *Tribolium castaneum***

## INTRODUCTION

The majority of animal species on the Earth are holometabolous insects, such as beetles (Coleoptera), flies (Diptera) or moths (Lepidoptera), whose transformation from wingless larvae to flying adults occurs by a transitory stage known as the pupa. This type of metamorphosis enabled higher specialization of the feeding larval stage and the exploitation of new niches, and, consequently, contributed to the Holometabola lineage success. How exactly is the switch from larval to pupal to adult developmental programs coordinated is far from being well understood.

Current models propose that two types of lipophilic hormones regulate the entry to metamorphosis. In the absence of the sesquiterpenoid juvenile hormone (JH), ecdysteroids initiate the pupal program, in which larval structures are transformed into imaginal ones and new pupal cuticle is deposited (Riddiford, 1994; Buszczak and Segraves, 2000; Gilbert et al., 2000; Thummel, 2001; Dubrovsky, 2005; Truman and Riddiford, 2007). The role of JH is anti-metamorphic, as in the presence of JH the larva cannot pupate but molts to another larva. Our knowledge about the molecular events underlying metamorphosis is primarily based on the *Drosophila* model. Unfortunately, fly development has largely lost dependence on JH, as constant exposure to JH cannot prevent entry to the pupal program (Riddiford and Ashburner, 1991; Restifo and Wilson, 1998). Thus, in response to an elevated ecdysteroid titer, *Drosophila* larval epidermis dies and the adult head and thorax, with appendages, develop from imaginal discs, while the abdominal epidermis proliferates from histoblasts (Madhavan and Schneiderman, 1977; Bayer et al., 1996b). Only at this later stage does ectopic JH interfere with *Drosophila* metamorphosis, most notably with the making of the adult abdomen (Postlethwait, 1974; Riddiford and Ashburner, 1991; Restifo and Wilson, 1998; Zhou and Riddiford, 2002). By contrast, both ecdysteroid and JH signals are necessary to coordinate metamorphosis in typical holometabolous larvae, whose polymorphic epidermal cells sequentially produce first larval, then pupal and finally adult structures (Riddiford, 1994), while internally growing imaginal discs, if any occur, give rise to appendages only, particularly the wings (Svacha, 1992).

Seminal studies in *Drosophila* have defined the ecdysteroid signaling pathway. The heterodimeric nuclear receptor consisting of EcR and Usp (Thomas et al., 1993; Yao et al., 1993) regulates primary ecdysteroid-response genes that encode transcription factors (Buszczak and Segraves, 2000). Of these transcription factors, E74 (Fletcher et al., 1995) and Broad-Complex (BR-C) are specifically required for metamorphosis. (*BR-C* has been designated as *br* by FlyBase, but, in keeping with nomenclature commonly used in the literature and to avoid confusion with the *BR-C* complementation group *br*, we use the original name.)

The *BR-C* gene encodes a Broad-Complex-Tramtrack-Bric-a-brac (BTB) domain with one of four alternatively spliced C2H2 zinc-finger motifs Z1-Z4 (DiBello et al., 1991; Bayer et al., 1996a). Mutants in the isoform-specific regions form three complementation groups (*br*, *rbp* and *2Bc*), and display both specific and partly overlapping defects in the differentiation of adult tissues and the death of larval ones; loss of the entire gene in *nonpupariating* (*npr1*) mutants blocks metamorphosis completely (Belyaeva et al., 1980; Kiss et al., 1988; Restifo and White, 1992; Fletcher and Thummel, 1995; Bayer et al., 1997).

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**Table 1. Primers and numbers of PCR amplification cycles used for RT-PCR analyses**

| Target sequence | Forward primer (5′-3′) | Reverse primer (5′-3′) | Number of cycles |
|---|---|---|---|
| *TcBR-C_core* | TCCCGACCACATCATCGGCAAC | CTCCCGTTCAGTGCTCGTGATG | 30 |
| *TcBR-C_Z1* | TTCCGATGTCAACCCTGCAAC | AACGTTCCTATGGTAAATGCTC | 30 |
| *TcBR-C_Z2* | TGCCAGCTGTGCGGTAAGGTG | GCGACTTGTGGTAAGTGTAGA | 32 |
| *TcBR-C_Z3* | TTCTCCTGCTACTACTCGCTC | AGTTTTGAGCAGACGTTTGAG | 30 |
| *TcBR-C_Z4* | CATCGCTGTGACGTCTGCG | GTCTGCGATGGTAAATACTGC | 30 |
| *TcBR-C_Z5* | CAGGACGGCGTTTTGCGACA | CTGACAAGCGAGTTCCGTGA | 30 |
| *TcMet* | GAAGCTTCAAGAGAGGAATATG | TTTCAACAGTTCCCTGGTCG | 28 |
| *Tcrp49* | TTATGGCAAACTCAAACGCAAC | GGTAGATGTGCTTCGTTTTG | 24 |
| *CpBR-C* | AACGTGGCGATATTGCAGCAC | CTTGACGTGGTCGCTTTTGTTC | 30 |
| *Cprp49* | TAAAGAGAAACTGGCGTAAACC | AATCCTGTGGGTAACATATGAC | 26 |

The function of *BR-C* is conserved at least in lepidopterans, as *BR-C Z4* from *Manduca sexta* partially rescues the *rbp* mutants (Bayer et al., 2003), and corresponding metamorphic defects result from *BR-C* RNAi in the silkmoth *Bombyx mori* (Uhlirova et al., 2003). However, because Diptera and Lepidoptera represent advanced and related insect orders, it is of interest to examine the role of *BR-C* in other holometabolans.

*BR-C* is an attractive target of the as yet poorly characterized JH signaling. Studies in *Manduca* (Zhou et al., 1998) and *Bombyx* (Reza et al., 2004) have shown that removal of the JH-producing corpora allata glands causes ectopic *BR-C* induction as well as precocious pupation, whereas exposure of larvae to JH prevents both *BR-C* transcription and pupation. *BR-C* is therefore thought of as the JH-dependent switch between larval and pupal programs (Zhou and Riddiford, 2002; Dubrovsky, 2005), but causal genetic evidence for this idea is missing. In the absence of a bona fide JH receptor, it is also unclear how JH might influence *BR-C* expression.

An excellent model with which to address these problems is the red flour beetle *Tribolium castaneum*, in which JH exerts its classical anti-metamorphic effect. We have recently shown that perturbed function of the *Tribolium* ortholog of the *Drosophila Methoprene-tolerant* (*Met*) gene causes larvae to metamorphose prematurely, before reaching their final instar (Konopova and Jindra, 2007). This phenotype is compatible with *Drosophila Met* mutation conferring resistance to JH and its mimic methoprene (Wilson and Fabian, 1986; Ashok et al., 1998), and this, together with the high-affinity binding of Met to JH (Shemshedini and Wilson, 1990; Miura et al., 2005), makes Met currently the best candidate for a JH receptor. Interestingly, *Met* and *BR-C* mutations interact genetically during *Drosophila* development (Wilson et al., 2006).

In this study, we use *Tribolium* and a neuropteran, lacewing *Chrysopa perla*, to show that the primary role of *BR-C* in directing the larval-pupal transition may have been present in primitive holometaboly. We propose that this role is a temporal coordination of tissues during the metamorphic process, which is at least partly achieved by Met-dependent JH regulation of *BR-C* expression.

## MATERIALS AND METHODS

### Isolation of *Tribolium* and *Chrysopa BR-C* cDNAs

Regions encoding the conserved BTB domain and the five zinc-finger domains were identified in the *Tribolium* genome database (www.bioinformatics.ksu.edu/BeetleBase) by using TBLASTN with *Drosophila* BR-C sequences. Sequence of the variable core region was obtained by RT-PCR (primers 5′-TCCAGGTGAATAGTGATTACG and 5′-GACTGTCTTTAACCTCGTTC) of the entire open reading frame of the *TcBR-C* isoform Z4 using total RNA from *Tribolium* prepupae. Sequences specific to the remaining four isoforms were obtained with a forward primer in the common core region and reverse primers in exons Z1-Z5 (see Table 1). The 5′ and 3′ cDNA ends were amplified by using the GeneRacer Kit (Invitrogen, Carlsbad, CA).

Partial sequence for a putative *Chrysopa* BR-C Z2 isoform was cloned from late-third (final) instar larvae by using touch-down, nested RT-PCR (Uhlirova et al., 2003), with primers mapping to the BTB and Z2 zinc-finger domains. PCR with a degenerate primer set 5′-AARWS-IACICCITGYAARCAYCC and 5′-CKISWRCARTAIACICKYTCRCA was followed by PCR with nested primers 5′-TAYCAYGGIGAR-GTIAAYGTNCA and 5′-TAYTCYTCYTGICKIWCNGCRTG. *BR-C* cDNA sequences from both species are available from GenBank (accession numbers EU200752-EU200758).

### Animals, RNAi and mutant analysis

Wild-type *Tribolium castaneum*, strain San Bernardino (obtained from G. Bucher, Georg August University, Goettingen) was reared at 32°C and staged as described (Konopova and Jindra, 2007). Under constant conditions, pupation takes place after seven or eight larval instars. Line *KS342* carrying a *piggyBac* insertion within the *TcBR-C* gene produced by the GEKU screen was kindly provided by S. Brown (Kansas State University, Manhattan, KS). An enhancer-trap line *pu11* that marks developing wings (Lorenzen et al., 2003; Tomoyasu and Denell, 2004) was a gift from Y. Tomoyasu (Kansas State University, Manhattan, KS). *Chrysopa perla* larvae were maintained at 24°C on a culture of live aphids (*Acyrthosiphon pisum*).

dsRNA of the indicated lengths (see Table 2) was prepared by using T3 and T7 MEGAscript kits (Ambion, Austin, TX). dsRNA concentrated up to 5 μg/μl was injected into the abdomen of $CO_2$ anesthetized *Tribolium* or *Chrysopa* larvae as described (Tomoyasu and Denell, 2004; Konopova and Jindra, 2007).

To determine the lethal phase in the *Tribolium KS342* line, we mated individual males carrying the *piggyBac* insertion (marked with EGFP) with wild-type females, and then crossed heterozygous *KS342*/+ males and females obtained from their progeny. Almost no lethality was observed in the embryos. The EGFP-positive larvae were reared and scored until adult stage. Whether larvae were homozygous for the *KS342* insertion was determined by PCR amplification of crude genomic DNA (Gloor et al., 1993) with primers 5′-GCAAAATTGCATCCGAGAAC and 5′-GTTTGCTTCACCGATATGAC flanking the site of *piggyBac* insertion.

### Methoprene treatment

Early *Tribolium* prepupae were injected with *TcMet* or *egfp* dsRNA and allowed to pupate. These RNAi pupae or intact pupae 0-4 hours after ecdysis were briefly dipped into 0.3 mM methoprene (VUOS, Pardubice, Czech Republic) in acetone, or into acetone alone (Konopova and Jindra, 2007). At the desired stage the pupae were subjected to mRNA expression analysis.

**Table 2. Lengths of dsRNA sequences used for RNAi**

| Target sequence | dsRNA length (bp) |
|---|---|
| *TcBR-C_core* | 586 |
| *TcBR-C_Z1* | 171 |
| *TcBR-C_Z2* | 176 |
| *TcBR-C_Z3* | 174 |
| *TcBR-C_Z4* | 169 |
| *TcBR-C_Z5* | 179 |
| *TcMet* | 564 |
| *CpBR-C* | 952 |
| *egfp* | 720 |

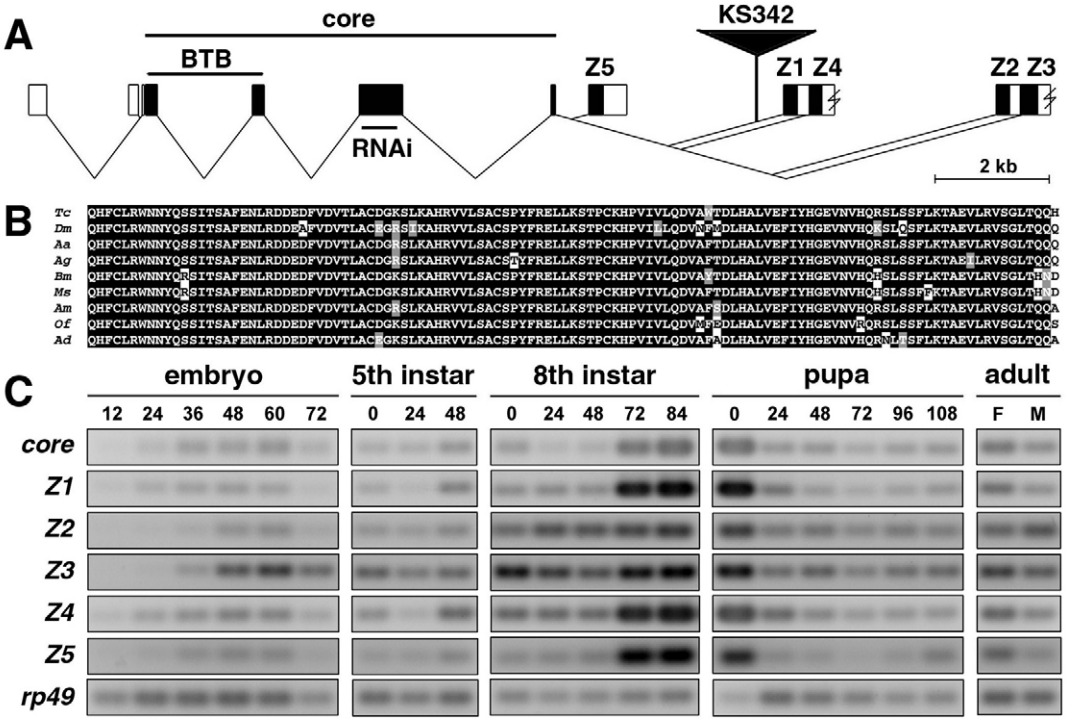


**Fig. 1. *TcBR-C* gene structure and mRNA expression.** (**A**) Exons of the common core region, including the BTB domain, are spliced to one of the five zinc-finger exons (Z1-Z5). Solid and open boxes indicate coding and untranslated mRNA regions, respectively. The open reading frame is preceded by two alternative noncoding exons. The region used for whole gene silencing is shown (RNAi). Mutant line *KS342* carries a *piggyBac* insertion 480 bp upstream of the Z1 exon. (**B**) Alignment of BR-C BTB domains. From top: *Tribolium castaneum, Drosophila melanogaster, Aedes aegypti, Anopheles gambiae, Bombyx mori, Manduca sexta, Apis mellifera, Oncopeltus fasciatus, Acheta domesticus*. (**C**) Total RNA from the indicated stages was subjected to DNase treatment and RT-PCR with primers for the core region or those specifically detecting the Z1-Z5 isoform transcripts. Numbers indicate hours since egg laying or ecdysis. F and M, 10-day old females and males, respectively. The prepupal stage begins at around 72 hours after the last larval ecdysis. Expression of *rp49* serves for control.

#### mRNA expression analysis

cDNA was prepared from 2 μg of total RNA isolated with TRIzol reagent (Invitrogen, Carlsbad, CA) and treated with DNase (Roche, Mannheim, Germany) as described (Konopova and Jindra, 2007). cDNA samples diluted 5-fold with water were subjected to standard PCR reactions with Taq DNA polymerase (Unis, Top-Bio, Prague, Czech Republic); the GC-rich Z3 cDNA was amplified with Phusion DNA polymerase (Finnzymes, Espoo, Finland). For primer sequences and cycle numbers see Table 1.

#### Scanning electron microscopy

Samples were fixed in 80% ethanol, postfixed with 1% osmium tetroxide, dehydrated in ethanol, critical-point dried, gold coated, and observed under a JEOL 6300 (Tokyo, Japan) scanning electron microscope.

## RESULTS

### *Tribolium BR-C* encodes conserved and novel protein isoforms

A putative *T. castaneum BR-C* ortholog (*TcBR-C*) has been identified in the *Tribolium* genome database by the presence of a conserved N-terminal BTB domain and of alternative C-terminal zinc finger DNA-binding domains (Fig. 1A). The BTB domain consists of 115 amino acids of which 90-97% residues are identical to those in BR-C BTB domains from other insects (Fig. 1B). We have isolated multiple transcripts of the gene, including two alternatively spliced 5′ untranslated regions and 3′ ends encoding five zinc finger domains alternatively spliced to the common core, comprising the BTB domain and a variable region (Fig. 1A). Four of the TcBR-C zinc finger motifs (Z1-Z4) show homology to those known in other insects (e.g. 84%, 91%, 100% and 93% amino acid identity with *Drosophila* BR-C Z1, Z2, Z3 and Z4, respectively). Moreover, the order in which the exons encoding the zinc finger domains follow within the genomic DNA (i.e. Z1, Z4, Z2 and Z3; Fig. 1A) is conserved among *Tribolium*, *Drosophila* and *Bombyx* (DiBello et al., 1991; Bayer et al., 1996a; Ijiro et al., 2004; Nishita and Takiya, 2004; Reza et al., 2004). Although in these species and in mosquitoes (Chen et al., 2004) no more than four isoforms have been found, an additional exon in *Tribolium* (Fig. 1A) predicts a fifth zinc-finger domain (Z5), whose sequence does not match any of the known BR-C proteins. All five isoforms produce transcripts with both of the alternatively spliced 5′ untranslated exons. The sequence homology together with the conserved microsynteny of the zinc finger-encoding exons demonstrates that the gene we have isolated is a *Tribolium BR-C* ortholog.

### Continuous *TcBR-C* expression peaks during the larva-pupa transition

To identify stages at which *TcBR-C* is expressed and potentially required, we analyzed cDNA samples prepared from embryos, fifth and eighth (final) instar larvae, pupae and adults. The transcript region common to all *TcBR-C* isoforms was detected continuously at moderate levels, with a strong peak rising at the onset of the prepupal (i.e. pharate pupal) stage and declining soon after ecdysis in early pupae (Fig. 1C, top row). To see whether there was any stage specificity in the presence or predominance of individual isoform mRNAs, we repeated RT-PCR with primer sets, each of which was able to amplify only one of the five zinc finger-encoding exons. Figure 1C shows that isoforms Z1, Z4 and Z5 were mainly responsible for

the sharp rise of total *TcBR-C* mRNA at 72 hours of the eighth instar, whereas isoforms Z2 and Z3 showed a more gradual increase. Nevertheless, all isoform-specific mRNAs could be detected at all examined stages. These data suggest that *TcBR-C* may play a role throughout development, although its upregulation during the onset of metamorphosis indicates its requirement for pupal differentiation.

### *TcBR-C* is required for morphogenesis of pupal characters

To determine the role of *TcBR-C* during postembryonic development, we injected *Tribolium* larvae with dsRNA directed against the variable common region (Fig. 1A) to silence all *TcBR-C* isoforms simultaneously. When fifth, sixth and final (seventh or eighth) instar larvae were injected, 100% of them ($n$=100) developed normally and underwent up to three successive larval molts, depending at which instar they had been injected. However, 98% of these animals eventually died during the prepupal stage at the end of the final larval instar (Fig. 2B,B′; see also Table S1 in the supplementary material). The penetrance of this phenotype was reduced to 64% ($n$=36) when third-instar larvae were injected, perhaps because of a weakening of the RNAi effect.

A prepupa is a pharate pupa in which larval cuticle has been apolysed and the newly deposited cuticle has attained pupal characters. However, removal of the apolysed larval cuticle from the arrested *TcBR-C(RNAi)* animals revealed recurrence of some larval features and disruption of the normal pupal morphogenesis (Figs 2, 3). These severely affected animals had an overall larval appearance with only rudimentary pupal wings (Fig. 2B′). The same lethal phenotype (Fig. 2C) was observed in 23% ($n$=260) of progeny produced by crossing beetles heterozygous for a *piggyBac* insertion *KS342* within *TcBR-C* (Fig. 1A). Similar to the effect of RNAi, the mutant prepupae possessed larval urogomphi and vestigial wings, and they lacked the pupal-specific cuticular structures called gin traps (Fig. 2C-G). As would be expected from the almost 1:3 ratio of dying to normally developing animals, the arrested prepupae were homozygous for the *KS342* mutation (see Materials and methods). *TcBR-C*$^{KS342}$ homozygotes had reduced levels of mRNAs encoding isoforms Z2 and Z3 (Fig. 2H), whose exons resided farther from the *piggyBac* insertion than exons Z1 and Z4 (Fig. 1A). We speculate that splicing of the Z2 and Z3 mRNA products might be compromised by the insertion, but further analysis is necessary. These results show that *BR-C* is required for pupal development.

Closer examination revealed that although cuticle in severely affected *TcBR-C(RNAi)* animals lacked the long larval setae, it remained smooth, without the microsculpture typical for pupae (Fig. 3A, part d; Fig. 3B, part d). The urogomphi retained their larval shape (Fig. 3A) and the gin traps were missing (Fig. 3C) in strongly affected *TcBR-C(RNAi)* prepupae. Other structures appeared to be less dependent on normal *TcBR-C* function and continued the pupal or adult program in arrested animals. For instance, distal abdominal segments differentiated pupal characters such as the genital papillae, but abnormalities were clearly visible (Fig. 3A, part d). The compound eyes developed several rows of ommatidia, although these were not evenly spaced as in normal pupae (Fig. 3D; Fig. 2D,E). Although the antennae lacked sensillae, they apparently developed towards the adult fate, with their club shape and clear separation of segments (Fig. 3E). Finally, the legs of *TcBR-C(RNAi)* animals lost the larval character as they possessed the double claws that normally develop in pupae and are typical for the adult leg (Fig. 3F). Slightly accelerated development was suggested by more distinctly separated tarsal segments and sharper claws in *TcBR-C(RNAi)* animals relative to normal pupae (Fig. 3F).

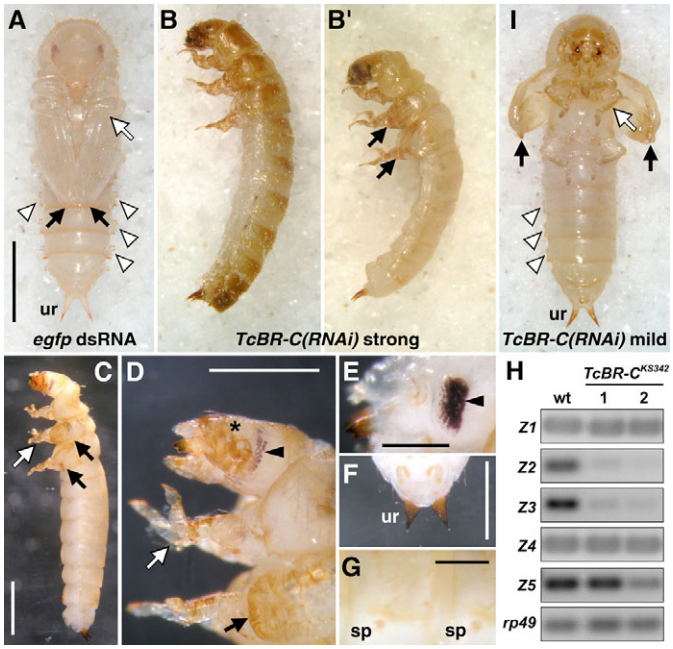


**Fig. 2. Loss of *TcBR-C* blocks *Tribolium* metamorphosis.** (**A**) A control pupa shows developed and elongated wings (black arrows), legs (white arrow) and gin traps (open arrowheads). (**B**) A prepupa that had been injected with the common-region *TcBR-C* dsRNA as a fifth-instar larva developed normally until it died at the end of the final instar. Upon removal of the apolysed larval cuticle the same animal displayed larval shape and rudimentary wings (**B′**, black arrows). (**C-G**) Essentially the same phenotype was found in dying animals homozygous for the *KS342 piggyBac* insertion that were freed from the larval cuticle. Note the vestigial wings and short legs (black and white arrows, respectively), defects in compound eyes (black arrowheads), thick antenna (asterisk), short larval urogomphi (F, ur) and missing gin traps (G) above abdominal spiracles (sp). (**H**) Total RNA from wild-type prepupae and from two sets of arrested *TcBR-C*$^{KS342}$ homozygous prepupae (two per sample) was subjected to RT-PCR. Note the reduced Z2 and Z3 mRNA levels in *TcBR-C*$^{KS342}$. Expression of *rp49* served as a control. (**I**) Lower doses of the common-region *TcBR-C* dsRNA allowed ecdysis into imperfect pupae with more developed yet abnormally short wings (black arrows in A through D), partially formed gin traps (white arrowheads), and short legs (white arrow). In B′ and C, larval cuticle could not be completely removed from the anterior head and distal legs. Scale bars: in A,1 mm for A-B′,I; C, 1 mm; D,F, 500 μm; E,G, 200 μm.

For a better insight into how *TcBR-C* effects the larval-pupal transition, we elicited a milder phenotypic response by diluting the dsRNA up to a 1000-fold. These lowered dsRNA doses were still lethal but allowed prepupae to ecdyse. All such treated animals had noticeably shortened and blistered wings and legs relative to control pupae (Fig. 2A,I). Compared with strong RNAi phenotypes, some pupal characters became more prominent. The animals developed gin traps, albeit aberrant (Fig. 2I; Fig. 3C, part e), their urogomphi were elongated, genital segments were nearly perfectly differentiated (Fig. 3A, part e), and the cuticle surface had a pupal-like microsculpture (Fig. 3B, part e). Except for the absence of sensory bristles, the antennae resembled the adult ones (Fig. 3E, part e). Conversely, legs with less prominent claws and segment borders suggested a weaker acceleration of the adult program than that observed with the strong RNAi effect (Fig. 3F).

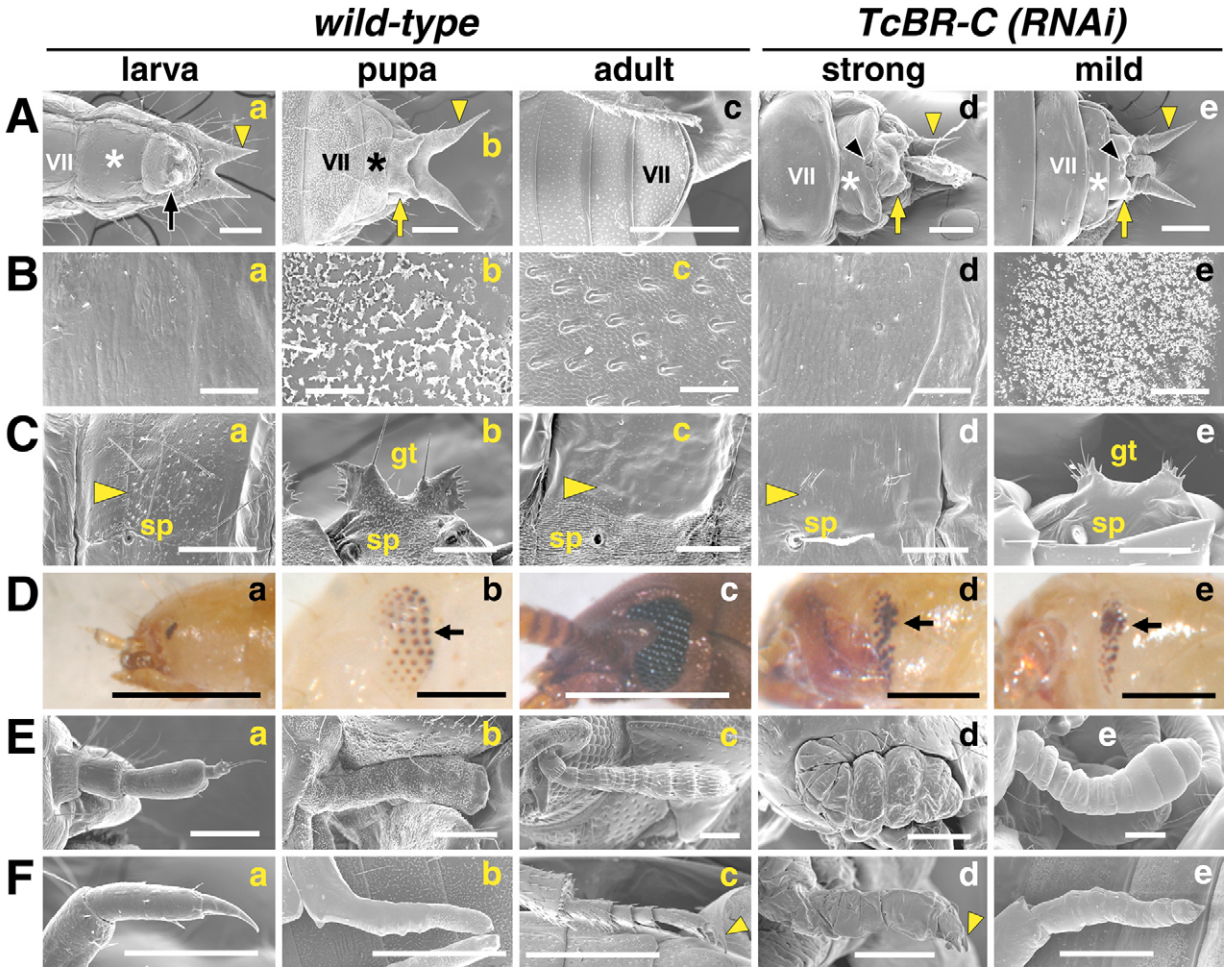


**Fig. 3. *TcBR-C* is necessary for proper differentiation of pupal characters.** Strong and mild phenotypes in animals that had been injected with *TcBR-C* dsRNA as fifth to seventh instar larvae are compared with wild-type. (**A**) Urogomphi in strongly affected animals are short, as in larvae, but elongated and forked, more like in pupae, upon mild *TcBR-C* RNAi treatment (a-e, yellow arrowheads; adults lack urogomphi). Larval pygopods (black arrow) disappear and genital papillae on the ninth abdominal segment develop (yellow arrows), although the eighth segment is abnormally enlarged after strong *TcBR-C* RNAi (asterisks). The female genital pore (black arrowheads), visible in its original intersegmental position (d) has migrated between the papillae in a mildly affected female (e). VII, the seventh abdominal sternum. Females shown in b, d and e. (**B**) Cuticle surface on abdominal sterna is smooth, as in larvae, upon strong *TcBR-C* RNAi, but shows the pupal-like surface in milder phenotypes (compare a with d, and b with e). The adult cuticle has rounded pits with sensillae (c). (**C**) Gin traps (gt) form above pupal abdominal spiracles (sp) but are absent in larvae, adults and in strong *TcBR-C(RNAi)* phenotypes (a-d; arrowheads mark presumptive gin trap positions). Weakly affected animals partially develop gin traps (e). (**D**) Larval stemmata (a) are replaced by compound eyes that differentiate during pupal stage (b). Ommatidia appear in *TcBR-C(RNAi)* animals, although they are irregularly spaced (arrows, compare d and e with b). (**E**) Antennae lose the larval character in *TcBR-C(RNAi)* animals, but, unlike those in pupae, develop the distal club and divisions between segments. (**F**) Separations between tarsal segments are clearer in strong *TcBR-C(RNAi)* than in pupae or mild phenotypes, and sharp double claws (arrowheads), similar to those of adults (c), are visible (d). Larval tarsi are unsegmented and bear a single claw (a). Anterior (A-D) and proximal (E,F) is to the left. Scale bars: 500 μm (Ac,Da,Dc,Fc); 200 μm (Aa,Ab,Ad,Ae,Ca,Db,Dd,De,Eb,Fa,Fb,Fe); 100 μm (Cb-e,Ea,Ec-e,Fd); 50 μm (Ba-e).

## Effects of *TcBR-C* isoforms

In *Drosophila*, some functions are shared by all BR-C proteins, whereas others are fulfilled by a specific isoform (Bayer et al., 1997). To see whether any unique functions apply to *Tribolium BR-C* isoforms, we injected larvae with dsRNA against each of the five zinc finger domains separately. Probably due to the limited sequence lengths, transcript levels of the targeted isoforms declined only partially (Fig. 4A). All larvae treated for a single isoform ecdysed into pupae displaying a degree of aberrancies increasing in the order of isoforms: Z5<Z1<Z4<Z3<Z2, with the most visible effect being the shortening of the wings and legs (Fig. 4; see also Fig. S1 in the supplementary material). These pupae developed into adults that either eclosed normally or died unable to ecdyse. Targeting of BR-C Z5 had no obvious effect. Interestingly, a simultaneous knockdown of Z1 and Z2 isoforms enhanced the short wing and leg phenotype, such that it resembled the mild effect of the common-region RNAi, including imperfect development of the gin traps (compare Fig. 2I with Fig. 4D). Thus, Z1 and Z2 might together be indispensable for wing elongation. Similarly, the apparent loss of Z2 and Z3 mRNAs in the *TcBR-C*$^{KS342}$ mutants could not be compensated for by the remaining *BR-C* products (Fig. 2H). Taken together, these results suggest at least some specific roles for the TcBR-C isoforms.

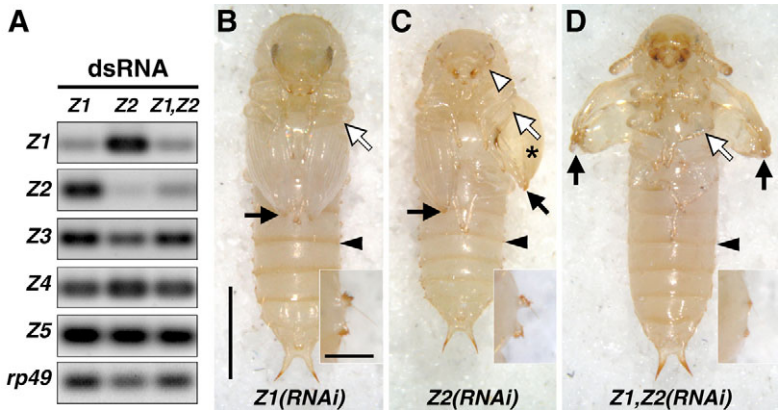


**Fig. 4. TcBR-C isoform-specific effects.** (**A**) Day 1 seventh-instar larvae were injected with indicated isoform-specific dsRNAs and subjected to RT-PCR as prepupae. Knocking down one isoform had no effect on the others. (**B-D**) Effects of knocking down TcBR-C isoforms. Black arrowheads mark the position to which wings normally extend in wild-type pupae (see Fig. 2A). Depletion of either Z1 (B) or Z2 (C) individually causes shortening of the wings (black arrows) and legs (white arrows), with the Z2 effect being stronger. Also note the aberrant head position (white arrowhead) and the blistered wing (asterisk). Combined double RNAi (D) enhanced the effect of isoform-specific dsRNAs on wings, legs and gin traps (insets), such that the aberrant pupae display the mild phenotypes of common-region *TcBR-C* RNAi (see Fig. 2I). Scale bars: in B, 1 mm for B-D; in insets, 200 μm.

## *TcBR-C* is induced during precocious pupation

Juvenile hormone (JH) is an anti-metamorphic signal that can block pupation; its removal causes precocious pupal development (Bounhiol, 1938; Wigglesworth, 1954; Nijhout, 1994). Precocious pupation of early-stage *Tribolium* larvae can be achieved by silencing of the *Methoprene-tolerant* gene (*TcMet*), which is necessary for JH signaling (Konopova and Jindra, 2007). If TcBR-C is indeed essential for the pupal program as suggested by our results, then *TcBR-C* mRNA should be upregulated in such untimely *TcMet(RNAi)* prepupae. To test this hypothesis, we injected early-fifth instar larvae with *TcMet* dsRNA and examined *TcBR-C* expression in resulting sixth-instar precocious prepupae. Figure 5 shows that the *TcBR-C* mRNA level in these *TcMet(RNAi)* prepupae was high relative to in control early-seventh instar larvae. The increase was comparable to that normally seen during the onset of metamorphosis (Fig. 5 and Fig. 1C). This result links precocious pupal development that ensues from a deficiency in JH signaling with upregulation of *TcBR-C*. It also supports the premise that *TcBR-C* is required for pupation, even if premature.

## JH regulates *TcBR-C* through *TcMet*

Similar to pupae of other insects, *Tribolium* pupae respond to exogenous JH or its mimic methoprene by deposition of a second pupal instead of adult cuticle (Konopova and Jindra, 2007). In *Manduca* and *Drosophila* this ectopic JH treatment induces *BR-C* expression (Zhou and Riddiford, 2002). We have used this effect to provide evidence that TcBR-C acts in JH signaling downstream of TcMet. First, we established that methoprene treatment indeed caused overexpression of *TcBR-C* in pupae. Figure 6A shows that, whereas in controls *TcBR-C* mRNA declines to its basal level after day 1 of normal pupal development, it is highly abundant throughout the pupal stage after methoprene application. As was shown previously (Konopova and Jindra, 2007), these methoprene-treated animals died as second-stage pupae.

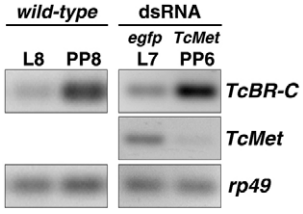


**Fig. 5. Loss of *TcMet* induces upregulation of *TcBR-C* mRNA in precocious prepupae.** RT-PCR shows that low *TcBR-C* expression in feeding day 1 eighth-instar larvae (L8) rises at the prepupal stage (PP8) 48 hours later (see also Fig. 1C). Injection of *TcMet* dsRNA to early-fifth instar larvae induced premature sixth-instar prepupae (PP6), which contained reduced *TcMet* mRNA and strongly elevated *TcBR-C* expression. Controls injected with *egfp* dsRNA and analyzed on day 1 of the seventh instar (L7) displayed a low *TcBR-C* mRNA level, typical for feeding larvae.

As the above effect of methoprene can be averted by the silencing of *TcMet* (Konopova and Jindra, 2007), we next checked whether *TcMet* RNAi also prevented the upregulation of *TcBR-C*. Early prepupae were injected either with *TcMet* or with control dsRNA and, after pupation, the animals were treated with methoprene. Pupae aged 24, 48, 72 and 96 hours were then subjected to RT-PCR. As shown by the 48-hour- and 96-hour-old pupae (Fig. 6B), *TcMet* depletion specifically prevented the induction of *TcBR-C* by methoprene. Consistent with our previous data (Konopova and Jindra, 2007), the methoprene-treated *TcMet(RNAi)* pupae produced adult beetles. This experiment demonstrates that TcMet is required for JH-induced upregulation of *TcBR-C* mRNA in *Tribolium* pupae. It also suggests that *TcBR-C* is a target of TcMet during JH-induced formation of the ectopic pupal stage.

## *BR-C* is required for metamorphosis in the lacewing *Chrysopa perla*

To explore whether the essential role of *BR-C* in pupal development might be common to other orders with less derived holometaboly, we chose to study the lacewing *Chrysopa perla* (Neuroptera). *Chrysopa* develops via three larval instars that are, unlike in *Tribolium*, easily discernible by distinct cuticle pigmentation and sensillation. Before pupation, *Chrysopa* larvae spin a cocoon from Malpighian tubules. We have isolated a cDNA fragment of a putative *Chrysopa perla BR-C* ortholog, hereafter referred to as *CpBR-C*. At its N terminus, this sequence shows 90% amino acid identity with the last 23 residues of the BR-C BTB domain; the C-terminal 23 amino acids match the *Drosophila* BR-C Z2 zinc-finger domain with 82% identity. The region between the two conserved domains shows little homology. Like in *Tribolium*, moderate *CpBR-C* mRNA levels were detected throughout embryogenesis (data not shown) and all three larval instars, with a marked expression peak before cocoon spinning at the end of final larval instar (see Fig. S2 in the supplementary material).

When first- and second-instar *Chrysopa* larvae were injected with *CpBR-C* dsRNA, no developmental defects were observed until the larval-pupal transition. Then, 95% ($n$=146) of the injected larvae arrested after the third instar at the prepupal stage, and 41% of them failed to complete or even initiate spinning their cocoons (see Table

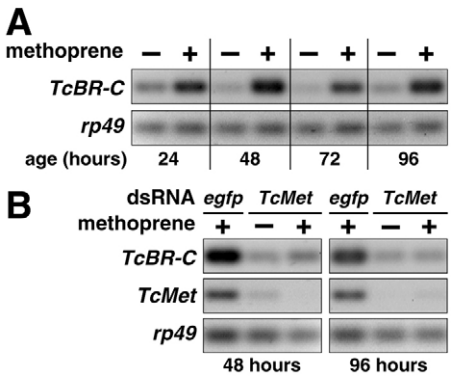


**Fig. 6. *TcBR-C* upregulation by methoprene requires *TcMet*.** (**A**) *Tribolium* pupae aged up to 1 hour after ecdysis were briefly dipped into 0.3 mM methoprene or its solvent (–) and tested (3-4 pupae per sample) for *TcBR-C* mRNA expression at the indicated times (adults normally emerge after 108-120 hours). Although *TcBR-C* expression in control pupae was low, similar to that in intact pupae of corresponding age (see Fig. 1C), it was markedly induced by methoprene. (**B**) Methoprene blocks adult development and its lethal effect is averted by *TcMet* knockdown (Konopova and Jindra, 2007). Early prepupae were injected either with *egfp* or *TcMet* dsRNA, and within four hours after pupal ecdysis were treated with methoprene or acetone. Shown are examples of *TcBR-C* mRNA expression in individual pupae aged 48 and 96 hours. Note that methoprene did not induce *TcBR-C* in *TcMet(RNAi)* pupae. Similar results were obtained with all examined pupae (at least eight for each treatment), aged either 48, 72 or 96 hours.

S2 in the supplementary material). The *CpBR-C(RNAi)* animals looked like imperfect pupae with very short wings and smaller compound eyes (Fig. 7). Although their cuticle pigmentation resembled that of pupae and lacked the long larval setae (Fig. 7), the cuticle showed typical larval thorns and was neither smooth, as in normal pupae, nor did it carry the long bristles seen in adults (Fig. 8A-D). The tarsi in *CpBR-C(RNAi)* animals became segmented as in pupae and adults, but pretarsi retained the larval character: compared with the pupal leg, they were narrow with hooked claws and ended with a long arolium similar to that found in larvae (Fig. 8E-H). In wild-type pupae, the long antennae pointed dorsally and were coiled on lateral sides (Fig. 7B), whereas in *CpBR-C(RNAi)* animals they were directed proximally and were twisted above the pupa-like mouthparts (Fig. 7F). Interestingly, differentiation of the compound eye in *CpBR-C(RNAi)* prepupae was more advanced relative to the smooth eye of control pupae, because it showed development of ommatidial lenses, although defective (Fig. 8I-L,J′-L′). Such premature eye differentiation probably resulted in holes instead of normal lenses (Fig. 8L). These results show that in *Chrysopa*, like in *Tribolium*, *BR-C* is required for pupation and plays important roles in the morphogenesis of certain pupal characters.

## DISCUSSION

### Role of BR-C in temporal tissue coordination during metamorphosis

In both *Tribolium* and *Chrysopa*, *BR-C* RNAi compromised the larval-pupal transition without affecting earlier development, regardless of the time of dsRNA injection. The *TcBR-C*$^{KS342}$ homozygotes died at the same stage. These data suggested that the moderate levels of *BR-C* mRNAs, detectable during premetamorphic stages in both species, had no essential role. This scenario would agree with the fact that zygotic *BR-C* function is not required in *Drosophila BR-C* null *nonpupariating* mutants until the onset of metamorphosis (Belyaeva et al., 1980; Kiss et al., 1988), and with recent data on *Tribolium* (Suzuki et al., 2008). However, as neither RNAi nor the likely hypomorphic *TcBR-C*$^{KS342}$ allele present a complete loss-of-function situation, we cannot exclude a possibility that *BR-C* plays some additional role, not visualized by our phenotypes. Importantly, the lethal phase correlates with a strong upregulation of *BR-C* expression. At least in beetles, this stage coincides with a peak of ecdysteroid titer that causes larvae to initiate prepupal development (Hirashima et al., 1995; Aribi et al., 1997).

In contrast to *Drosophila npr1* mutants, metamorphosis was not completely blocked by *BR-C* deficiency in *Tribolium* or *Chrysopa*. Instead the arrested prepupae showed a blend of larval, pupal, and partially even adult features. Based on the absence of the pupal-specific gin traps in *Tribolium* and on the surface microsculpture, the cuticle was apparently larval in both species, thus confirming the requirement of *BR-C* for the pupal commitment of the epidermis (Zhou et al., 1998; Zhou and Riddiford, 2002). Interestingly,

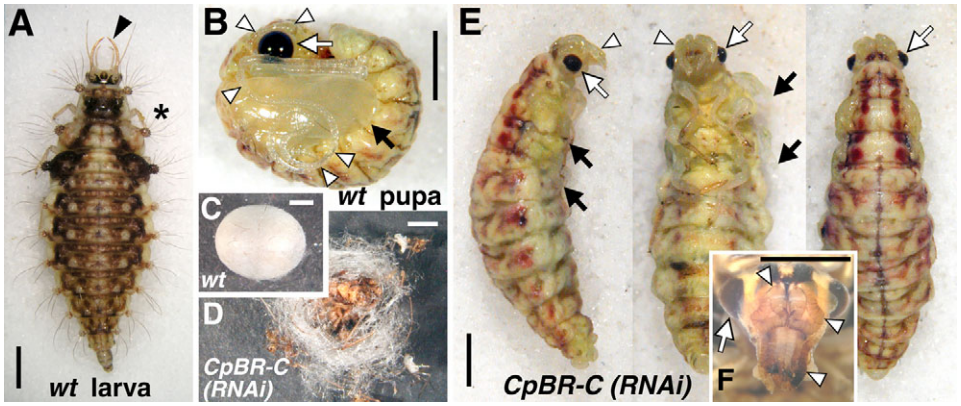


**Fig. 7. *BR-C* knockdown impairs cocoon spinning and pupal differentiation in *Chrysopa*.** (**A-C**) Normal final (third) instar larva (A) spins a cocoon (C), in which it ecdyses into a pupa (B). (**D-F**) Injection of *CpBR-C* dsRNA into larvae kills them as prepupae; some of these larvae are unable to initiate or complete spinning of their cocoons (D). Upon removal of the larval cuticle (E,F), an arrested animal reveals pupal pigmentation, the development of compound eyes (white arrows) and short wings (black arrows), whereas typical larval mouthparts (stylets; A, black arrowhead) and long setae (asterisk) are missing. In contrast to in pupae, antennae are twisted above mouthparts, with their end oriented toward the anterior in *CpBR-C(RNAi)* animals (compare white arrowheads in panels B,E and F). Scale bars: 1 mm.

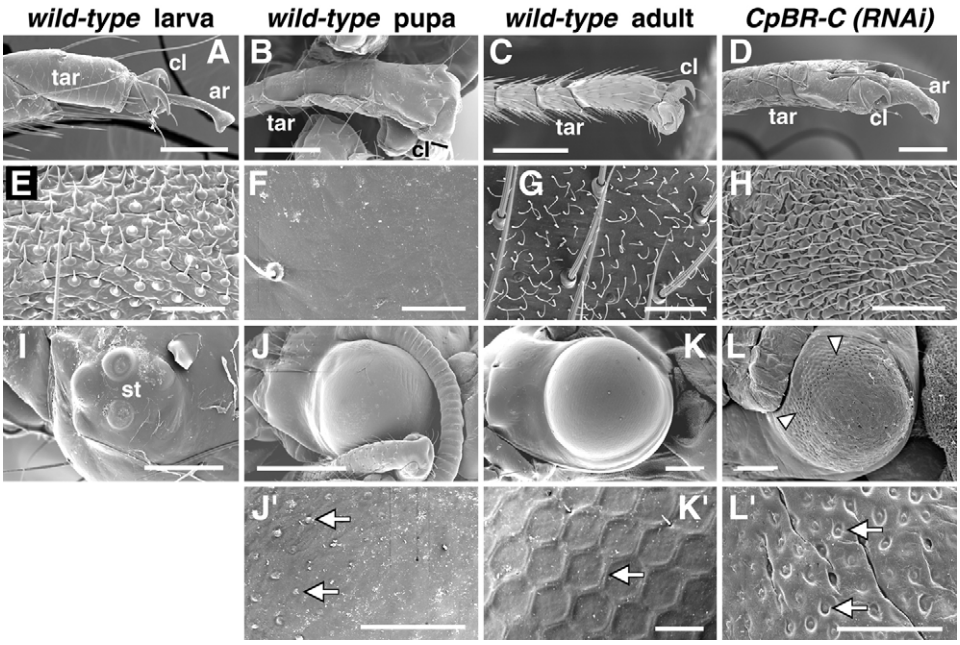


**Fig. 8. *CpBR-C* RNAi prevents proper differentiation of pupal characters.** *Chrysopa* larvae were injected with *CpBR-C* dsRNA at the end of the first instar and, after they arrested at the prepupal stage, the larval cuticle was removed. Examples of their heterochronic phenotypes are compared with the normal larval, pupal and adult situation. (**A-D**) Tarsi (tar) of *CpBR-C(RNAi)* individuals (D) have distinct borders between segments, as in pupae and adults, whereas their distal tip (pretarsus) retains the larval character and is narrow, with a long arolium (ar). Claws (cl) are large and hooked as in larvae or adults. (**E-H**) Dorsal abdominal cuticle with short thorns resembles that of the larva. The thorns in *CpBR-C(RNAi)* (H) are densely packed because the animal had been compressed in its old larval cuticle. (**I-L′**) Instead of larval stemmata (st), *CpBR-C(RNAi)* animals develop compound eyes, also present in pupae and adults. Compared with pupa, eye differentiation is more advanced, as indicated by the formation, although incomplete, of ommatidial lenses (arrows, compare J′ with L′). Also note the perforations (arrowheads) on the *CpBR-C(RNAi)* eye (L). Scale bars: 500 μm (J); 200 μm (B,C,K); 100 μm (A,D,I,L); 50 μm (E-H,J′,L′); 20 μm (K′).

although the thorny cuticle in *Chrysopa BR-C(RNAi)* animals was distinctly larval, similar to in *Tribolium*, the body pigmentation resembled that of pupae. We cannot be sure whether this mixed character of the epidermis might be due to persisting *CpBR-C* function, or might be because *CpBR-C* is not necessary for the pupal pigmentation.

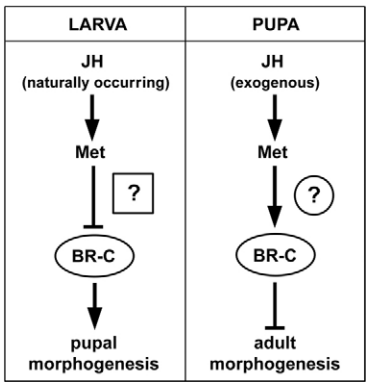


**Fig. 9. Model for functioning of BR-C in *Tribolium* metamorphosis.** In young larvae, naturally occurring JH blocks pupal differentiation by repressing *BR-C*. JH is absent in early pupae, and its addition blocks adult morphogenesis by causing ectopic *BR-C* activation and death after a supernumerary pupal cuticle deposition. As both effects of JH on *BR-C* expression require Met, unknown stage-specific factors must modulate Met function.

Pupal characters in *BR-C(RNAi)* animals included rudimentary wings. In particular, the weak phenotypes in *Tribolium* (produced with either isoform-specific or diluted common-core dsRNAs) revealed that wing elongation was highly sensitive to BR-C depletion. A similar effect of *BR-C* RNAi was described for pupal appendages in *Bombyx mori* (Uhlirova et al., 2003). *BR-C* silencing prevented the gradual wing enlargement even in larvae of the hemimetabolous milkweed bug *Oncopeltus fasciatus* (Erezyilmaz et al., 2006). Imaginal discs fail to elongate in *Drosophila br* mutants with disrupted BR-C Z2 function (Kiss et al., 1988; DiBello et al., 1991; Bayer et al., 1997). The short legs and wings are not due to insufficient proliferation of the disc cells but are due to their inability to change shape in response to the ecdysteroid (von Kalm et al., 1995). This cell shape change requires cytoskeletal components whose mutations enhance the effect of *br* (Gotwals and Fristrom, 1991; Ward et al., 2003). The rudimentary wings, present even in animals most severely affected by *TcBR-C*$^{KS342}$ mutation or by RNAi, suggest that cell shape changes, rather than cell proliferation may be disrupted by the loss of BR-C in *Tribolium* as well. Growing wings marked by EGFP in arrested beetle prepupae (see Fig. S3 in the supplementary material) support this idea. The legs in *Tribolium BR-C(RNAi)* animals were short also but were distally specified as pupal with two tarsal claws. By contrast, the arrested *Chrysopa* prepupae retained pretarsi with the larval-specific elongated arolium, thus suggesting a stronger requirement for *BR-C* function in the *Chrysopa* leg.

Except for small deviations, gross morphology of *Tribolium* genital segments with the pupal genital papillae was pupal in *BR-C(RNAi)* animals (see Fig. 3A). In addition, the larval-pupal

transformation of the visual system was initiated, as larval stemmata were replaced with ommatidia of the compound eyes. However, as in *Drosophila* (Brennan et al., 2001), *TcBR-C* was important for compound eye differentiation. These observations suggested that not all aspects of pupal development were completely blocked by BR-C depletion.

While the above described structures were retarded in their development in *BR-C(RNAi)* animals, others appeared accelerated in their development towards the adult state, although none could be unambiguously defined as adult. For instance, the antennae in *Tribolium* or the compound eyes in *Chrysopa* resembled their adult counterparts, but in fact were intermediates between pupal and adult organs. These heterochronic phenotypes suggest that BR-C may not only be a pupal specifier (Zhou and Riddiford, 2002), but rather a temporal coordinator of the extensive morphogenesis in diverse tissues during metamorphosis.

*Drosophila* organs require a temporally regulated balance between both inductive and repressive BR-C functions, represented by the individual isoforms (Karim et al., 1993; Crossgrove et al., 1996; Mugat et al., 2000). We therefore see two alternative explanations for the heterochronically advanced phenotypes. First, these structures may require BR-C to repress precocious adult morphogenesis in them, but the inductive BR-C function is dispensable for development beyond larval state. Consequently, loss of BR-C accelerates their development. Second, if both functions are required but the repressive one is more sensitive to reduced BR-C dose, then the inductive function will prevail under an incomplete BR-C knockdown. We favor the first alternative, because progression beyond the pupal stage seems to depend on *BR-C* downregulation (Zhou and Riddiford, 2002) (this work).

## Regulation of *BR-C* by Met-dependent JH signaling

Periods of JH absence are required first in larvae to initiate the pupal program, and later in pupae to exit it. BR-C in both cases promotes the pupal fate (Zhou and Riddiford, 2002), and therefore JH must regulate *BR-C* differently in larvae and in pupae. In lepidopteran (Zhou et al., 1998; Reza et al., 2004), as well as in *Tribolium* (Suzuki et al., 2008) larvae, JH prevents *BR-C* expression until the onset of metamorphosis, and presumably that is how JH prevents pupal differentiation. Conversely, removal of the JH source (allatectomy) causes both *BR-C* misexpression and precocious pupal development. In pupae, ectopic JH induces *BR-C* (Zhou et al., 1998; Zhou and Riddiford, 2002; Reza et al., 2004; Wu et al., 2006), and in many insects, including *Tribolium* (Konopova and Jindra, 2007), such JH application causes reiteration of the pupal stage. In *Drosophila*, *BR-C* misexpression alone is sufficient to inhibit adult cuticle formation (Zhou and Riddiford, 2002). *BR-C* is therefore a prime target of JH signaling, but how JH regulates *BR-C* expression is unknown.

We showed here that precocious pupation, triggered by interference with the putative JH receptor Met, coincided with precocious *TcBR-C* mRNA increase in the sixth instar. Thus, disrupted JH signaling induced *TcBR-C* similarly to allatectomy in lepidopteran larvae (Zhou et al,. 1998; Reza et al., 2004). As expected, *TcBR-C* not only marked but also was necessary for the untimely pupation, as *TcMet*; *TcBR-C* double-RNAi resulted in a phenotype similar to *TcBR-C* RNAi alone, i.e. entry to a lethal prepupal stage, except one or two instars too early (data not shown). Therefore, although the metamorphic program could be prematurely induced by silencing of *TcMet*, it could not be completed without *TcBR-C*. However, loss of *Met* has been shown to worsen the effect of *BR-C* mutations in *Drosophila*, without altering *BR-C* expression (Wilson et al., 2006). This again might reflect the different response to JH in the fly.

The evidence that *TcMet* is required for regulation of *TcBR-C* came from pupae, where the JH mimic methoprene induced *TcBR-C* mRNA, but not after *TcMet* knockdown. This result places *TcBR-C* downstream of *TcMet* in JH signaling. Importantly, the averting of ectopic *TcBR-C* expression by *TcMet* RNAi also rescued the methoprene-treated animals from repeating the pupal stage and allowed them to become adult (Konopova and Jindra, 2007). Together, these findings suggest that, similar to in *Drosophila* (Zhou and Riddiford, 2002), downregulation of *BR-C* is required to exit the pupal state in *Tribolium*.

We propose the following model for BR-C function in holometabolan metamorphosis (Fig. 9). In larvae, JH acts through Met to prevent *BR-C* induction until the final instar, when JH decline relieves the repression, and BR-C coordinates pupal morphogenesis. Loss of BR-C function causes both retardation and acceleration of development in diverse epidermal tissues, thus producing a mix of larval-, pupal- and adult-like features. In early pupae, low JH titer normally allows *BR-C* expression to drop, which is necessary for proper adult differentiation. Exogenous JH, again acting via Met, causes *BR-C* misexpression, which in turn promotes another round of pupal, instead of adult, development. Whether Met regulates *BR-C* expression directly, and what determines whether *BR-C* will be repressed or activated requires further work.

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### Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/3/559/DC1

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**Table S1. RNAi against all *BR-C* isoforms causes prepupal stage arrest in *Tribolium***

| dsRNA | Instar injected | *n* | Stage of developmental arrest | | | | Normal adults |
|---|---|---|---|---|---|---|---|
| | | | Larva | Prepupa | Defect. pupa | Pupa | |
| *TcBR-C* | L3 | 36 | – | 23 | – | 8 | 5 |
| | L5 | 28 | – | 28 | – | – | – |
| | L6 | 38 | – | 38 | – | – | – |
| | L7* | 34 | – | 32 | 2 | – | – |
| *egfp* | L3 | 27 | – | 1 | – | – | 26 |
| | L5 | 6 | – | – | – | – | 6 |
| | L6 | 8 | – | – | – | – | 8 |
| | L7* | 37 | 1 | – | – | – | 36 |

*Final or penultimate larval instar.

**Table S2. *BR-C* RNAi causes prepupal stage arrest in the lacewing *Chrysopa perla***

| dsRNA | Instar injected | *n* | Stage of developmental arrest | | | | | | Normal adults |
|---|---|---|---|---|---|---|---|---|---|
| | | | L1 | L2 | Prepupa no cocoon | Prepupa weak cocoon | Prepupa normal cocoon | Pupa | |
| *CpBR-C* | Early L1 | 34 | – | 1 | 6 | 6 | 16 | 4 | 1 |
| | Late L1 | 63 | – | – | 29 | 1 | 33 | – | – |
| | Early L2 | 26 | – | – | 4 | 2 | 18 | 2 | – |
| | Late L2 | 12 | – | – | 2 | – | 10 | – | – |
| | Early L3* | 11 | – | – | 4 | 2 | 5 | – | – |
| *egfp* | Early L1 | 7 | – | – | – | – | – | – | 7 |
| | Late L1 | 36 | – | – | 1 | – | 1 | 1 | 33 |
| | Early L2 | 6 | 1 | – | – | – | – | – | 5 |
| | Early L3* | 5 | – | – | – | – | – | – | 5 |

*Final larval instar.