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The role of Broad in the development of *Tribolium castaneum*: implications for the evolution of the holometabolous insect pupa

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The evolution of complete metamorphosis in insects is a key innovation that has led to the successful diversification of holometabolous insects, yet the origin of the pupa remains an enigma. Here, we analyzed the expression of the pupal specifier gene *broad* (*br*), and the effect on *br* of isoform-specific, double-stranded RNA-mediated silencing, in a basal holometabolous insect, the beetle *Tribolium castaneum*. All five isoforms are weakly expressed during the penultimate instar and highly expressed during the prepupal period of the final instar. Application of hydroprene, a juvenile hormone analog, during the penultimate instar caused a repeat of the penultimate *br* expression patterns, and the formation of supernumerary larvae. Use of dsRNA against the *br* core region, or against a pair of either the *br-Z2* or *br-Z3* isoform with the *br-Z1* or *br-Z4* isoform, produced mobile animals with well-differentiated adult-like appendages, but which retained larval-like urogomphi and epidermis. Disruption of either the *br-Z2* or the *br-Z3* isoform caused the formation of shorter wings. Disruption of both *br-Z1* and *br-Z4* caused the appearance of pupal traits in the adults, but disruption of *br-Z5* had no morphological effect. Our findings show that the *br* isoform functions are broadly conserved within the Holometabola and suggest that evolution of *br* isoform expression may have played an important role in the evolution of the pupa in holometabolous insects.

KEY WORDS: Metamorphosis, Broad, Juvenile hormone, Pupation, *Tribolium*

INTRODUCTION

The mechanisms behind major macroevolutionary events, such as the evolution of key innovations, remain a crucial issue in evolutionary biology. Key innovations are novel traits that perform novel functions that allow the adaptive radiation of species (Mayr, 1963). These events are rare and thus their evolutionary origins remain poorly understood.

The evolution of complete metamorphosis (holometaboly) in insects is a key evolutionary innovation that has contributed to their success (Yang, 2001). In holometabolous insects, the three life history stages, larva, pupa and adult, have morphologies that are highly adapted to the ecological pressures encountered and that have little or no resemblance to each other. Holometabolous insects are thought to have evolved from hemimetabolous insects, which have only two life history stages, the nymph and the adult. The hemimetabolous insects are direct developers in that the nymphs resemble the adults except for the genitalia and wings, which develop as everted pads or buds during nymphal growth and molting. The evolutionary origin of the three morphologically distinct, life-history stages of holometabolous insects remains an enigma. A developmental basis for this major evolutionary event may shed light onto how major key innovations evolve.

Various theories have been proposed to explain the evolutionary origin of holometaboly (Berlese, 1913; Heslop-Harrison, 1958; Hinton, 1963; Truman and Riddiford, 1999; Erezyilmaz, 2006). One current theory based on a proposal by Berlese (Berlese, 1913) and on present knowledge of the endocrine regulation of embryonic development is that the holometabolous larva corresponds to the hemimetabolous embryonic stage called the pronymph (Truman and Riddiford, 1999). According to this theory, in holometabolous insect embryos, juvenile hormone (JH), which is secreted earlier than in hemimetabolous embryos, truncates patterning cascades and promotes precocious differentiation, thereby resulting in a novel larval form. Only when JH declines in the final larval instar does extensive morphogenesis resume and lead to differentiation of the pupa (Truman and Riddiford, 2007).

Although a shift in JH titers may explain the origin of larval form, this theory implies that the holometabolous pupa and the hemimetabolous nymph are homologous developmental stages. A change in the embryonic JH titer alone cannot explain the formation of the pupal morph, which, with its immobile and compact morphology, shares little resemblance to the mobile feeding nymphs. This implies that a second major developmental reorganization must have occurred during the evolution of the pupa. This paper examines the developmental regulation of pupal morphology in beetles (Coleoptera), which diverged nearly 300 million years ago from other higher insects, such as Diptera and Lepidoptera (Kristensen, 1999).

A major gene involved in specifying pupal development is the BTB/POZ (Bric-a-brac, Tramtrack, Broad-complex/POx virus Zinc finger) domain transcription factor *broad* (*br*), which has been shown to specify pupal fates in *Drosophila melanogaster* (Meigen) (Zhou and Riddiford, 2002) and whose expression correlates with the timing of pupal commitment in the tobacco hornworm, *Manduca sexta* (Linnaeus) (Zhou et al., 1998; Zhou and Riddiford, 2001). The *Drosophila* and *Manduca br* genes encode four different alternatively spliced isoforms of zinc-finger transcription factors, which share a common BTB core domain (DiBello et al., 1991; Bayer et al., 1996a). During the last larval instar, each of the isoforms is expressed in a spatiotemporo-specific manner that

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coordinates the onset of major metamorphic changes during pupal development (Emery et al., 1994; Bayer et al., 1996a). Functional analyses have confirmed the role of *br* in specifying pupal fates: misexpression of the *br* isoform Br-Z1 during both larval and adult development in *Drosophila* leads to the appearance of pupal-specific products during the respective molts (Zhou and Riddiford, 2002).

In *Drosophila* and *Manduca*, the high expression of *br* is confined to the time of metamorphosis to the pupa, and is regulated by hormonal inputs. Ecdysone [used as a general term here; see Riddiford et al. (Riddiford et al., 2000)] and JH play an important role in coordinating the expression of *br*. *br* is one of the early ecdysone response genes whose expression activates the tissue-specific late ecdysone response genes during prepupal development (Karim et al., 1993; Bayer et al., 1996b). In *Manduca*, the initial expression of *br* is prevented in the presence of JH (Zhou et al., 1998; Zhou and Riddiford, 2001). During the pupal stage, *br* expression declines to undetectable levels; but application of JH before the onset of the adult molt leads to the upregulation of *br* and the subsequent formation of a second pupal cuticle in *Manduca* and in the abdomen of *Drosophila* (Zhou and Riddiford, 2002). Thus, prior to the initial onset of *br* expression, JH must decline; once *br* is expressed, JH can maintain its expression in response to ecdysone.

In the hemimetabolous insect *Oncopeltus fasciatus* (Dallas), *br* is expressed throughout nymphal development and is involved in the morphogenetic changes that occur between the different nymphal instars (Erezyilmaz et al., 2006). *br* only disappears when ecdysone rises in the absence of JH in the final instar. A considerable evolutionary gap exists between the highly derived Lepidoptera/Diptera and the hemimetabolous insects. Thus, in order to understand the evolution of metamorphosis, we chose to examine the role of *br* in a more basal holometabolous insect, the flour beetle *Tribolium castaneum* (Herbst). We find that removal of *br* by injection of dsRNA has no apparent effect on the gross morphology of the final larval molt. But this removal disrupts the normal larval-pupal transformation, resulting in the formation of an individual with larval and adult characteristics.

MATERIALS AND METHODS

Animals

Tribolium was obtained from the USDA ARS Biological Research Unit, Grain Marketing & Production Research Center, Manhattan, Kansas (gift of Dr Richard Beeman). The beetles were reared on organic wheat flour containing 5% nutritional yeast. Stocks were maintained in a 26.5°C walk-in incubator in small plastic containers under a long-day photoperiod regimen (17 hours light:7 hours dark).

Hormonal application

The JH analog (JHA) 95% S-hydroprene (SDS Biotech, Tokyo, Japan) was diluted in acetone. Diluted solution (0.5 μl) containing different concentrations of hydroprene was applied topically onto the dorsal side of mid-sixth instar larvae or to pupae less than 4 hours after ecdysis. The same amount of acetone was applied to control larvae or pupae.

Amplification of cDNA

Using the *Tribolium* Genome Base (http://www.bioinformatics.ksu.edu/BeetleBase/), primers were designed to amplify regions of interest. For the *br* core region, a 250 bp fragment downstream of the BTB domain was amplified using the forward primer TCGGCAACAACAACAATAAC and the reverse primer CATCGGTTCGCTCTTCAC. For the isoform-specific fragments, the following primer combinations were used: TGTGGACGAGTTCCGATG (Z1 forward) and TCCTATGGTAAATGCTCTTGTG (Z1 reverse); GTCGCCCAAGAAGGTGT (Z2 forward) and CGACTTGTGGTAAGTGTAGATGTG (Z2 reverse); CGCACCTTCTCCTGCTACT (Z3 forward) and GCGTCGTGAGCGAGTTTT (Z3 reverse); TTGAGTCTTCCACTTCCACTGA (Z4 forward) and GCGATGGTAAATACTGCGATG (Z4 reverse); and ACGGTTTTGGTCCCTCCA (Z5 forward) and CTCCGATGGCTGACAAGC (Z5 reverse).

mRNA isolation

Larvae from penultimate and final instars, first day pupae and pharate adults were flash-frozen in liquid nitrogen and stored at –80°C until use. Larvae and pupae were dissected in phosphate-buffered saline [PBS; 0.0038M NaH_2PO_4, 0.0162 M Na_2HPO_4, 0.15 M NaCl (pH 7.4)], and the fat body and gut were removed. After Trizol (Invitrogen, Carlsbad, CA, USA) and chloroform extraction, RNA was DNAse-treated and precipitated in isopropanol. cDNA was synthesized from 1 μg of total RNA using the cDNA Synthesis Kit (Fermentas, Hanover, MD), according to the manufacturer's instructions, and stored at –20°C until use.

RT-PCR

RT-PCR reactions to examine the expression profiles of *br* isoforms were conducted using the forward primer from the *br* core region and each of the isoform-specific reverse primers (same as those given above for *br-Z1*, *br-Z2*, *br-Z3* and *br-Z5*; GCCTTTTCAGAGTGAGTTTGGT for *br-Z4*), under the following PCR cycle conditions: 30 seconds at 94°C, 30 seconds at 55°C and 1.5 minutes at 72°C. Cycle numbers used were: 38 cycles for *br-Z1*; 40 for *br-Z2*; 34 for *br-Z3*; 32 for *br-Z4*; and 35 for *br-Z5*. In addition, 37 cycles were used for *ribosomal protein subunit3* (*rps3*) (Mahroof et al., 2005), a control for the variation in the amount of cDNA loaded. For all PCR reactions, the mixture was held at 94°C for 5 minutes before starting the PCR cycles, and then held at 72°C for 5 minutes before being cooled to 4°C.

The samples for each treatment were run on the same gel and visualized under UV light. Pictures were taken with a Polaroid camera (Polaroid, Waltham, MA, USA), and levels were adjusted for the entire gel picture using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

dsRNA synthesis and injection

For double-stranded RNA (dsRNA)-mediated silencing of *br*, dsRNA was created as follows. cDNA amplified with the primers listed above was cloned using either the Topo TA vector (Invitrogen) or the PGEM vector (Promega, Madison, WI, USA). Sequencing confirmed the identity of clones with the following insertions: 251 bp, 177 bp, 184 bp, 121 bp, 196 bp and 120 bp fragments for the core region, *br-Z1*, *br-Z2*, *br-Z3*, *br-Z4* and *br-Z5* isoforms, respectively. Each of the strands of the dsRNA was synthesized using the MEGAscript Kit (Ambion, Austin, TX); strands were annealed as described (Hughes and Kaufman, 2002).

Because *Tribolium* larvae have variable number of instars, we collected both penultimate and final instars for dsRNA injection. Approximately 1 μg (0.5 μl) of dsRNA was injected into either day 2 penultimate or final instar larvae using a 10 μl glass capillary needle connected to a syringe. Controls received the same volume of either DEPC water or dsRNA of the bacterial *ampicillin-resistance* gene (*amp*^{r}) (plasmid obtained from Dr Takashi Koyama, our laboratory). Larvae were then kept at 30°C until processed. Pupal phenotypes were examined one day after pupal ecdysis, and adult phenotypes were examined once the adult cuticle became completely tanned.

RESULTS

Structure of *br* isoforms in *Tribolium castaneum*

The *Tribolium castaneum br* gene was identified in the *Tribolium* Genome Base (http://www.bioinformatics.ksu.edu/BeetleBase/), and we found that the BTB domain in the *br* core region shares high amino acid sequence conservation with the BTB domain of the *Manduca* (Zhou et al., 1998) and *Drosophila br* genes (DiBello et al., 1991) (Fig. 1). There were five zinc-finger domains, four of which share high amino acid sequence similarity with the *Drosophila br* zinc-finger isoforms: Br-Z1 (85%), Br-Z2 (85%), Br-Z3 (97%) and *Br-Z4* (90%). The *Tribolium* Br-Z2, Br-Z3 and Br-Z4 isoforms also shared high sequence similarity with *Manduca* Br-Z2 (93%), Br-Z3 (98%) and Br-Z4 (82%) isoforms, respectively. The fifth isoform was found to contain two zinc fingers that had low

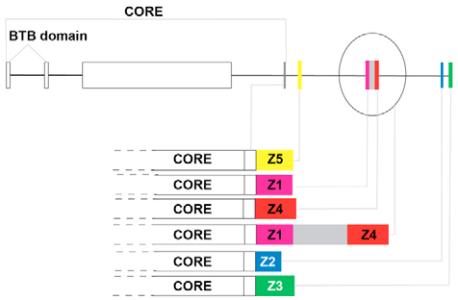

Fig. 1. The structure of the *Tribolium br* gene inferred from the genome and the isolated mRNA of *br* isoforms identified in the present study. Five zinc-finger domains (*br-Z1* through *br-Z5*) were found in close proximity to the *br* core region. mRNA of the *br* isoforms consisted of each of these isoform-specific domains alternatively spliced to the core region. An additional isoform was found that consisted of the *br* core region spliced to the *br-Z1* and *br-Z4* zinc-finger domains with an intron between them.

sequence similarity to the *br* isoforms in *Drosophila* (39%, 36%, 43% and 35% for Br-Z1, Br-Z2, Br-Z3 and Br-Z4, respectively), *Manduca* (39%, 43% and 35% for Br-Z2, Br-Z3 and Br-Z4, respectively) and *Bombyx* (43%, 39%, 43% and 35% for Br-Z1, Br-Z2, Br-Z3 and Br-Z4, respectively) and will be called Br-Z5 in the current paper. The Br-Z5 isoform was also different from the other *br* isoforms in that the lysine (K) in the predicted amino acid sequence (LKRH) within the 5′ zinc-finger domain in the other isoforms has been replaced by glutamine (Q).

To identify the structure of the isoform-specific mRNA sequences, a forward primer for the *Tribolium br* core region and a reverse primer for each of the five isoforms were used to PCR-amplify mRNA transcripts of each of the isoforms. For each of the *br-Z1*, *br-Z2*, *br-Z3* and *br-Z5* isoforms, a single band was observed following gel electrophoresis (data not shown), and sequencing the transcripts showed that each consisted of the *br* core region linked to one of the isoforms (Fig. 1). For the *br-Z4* isoform, two bands were seen. One had a similar transcript structure to the other isoforms (referred to as the *Z4* isoform). The second was longer and consisted of the core region linked to the *br-Z1* and *br-Z4* isoforms with an intron between the two isoforms (referred to as the *Z1/4* isoform). There is a stop codon before the intron, and the presence of the intron results in a frame shift of the Z4 protein. Thus, it is likely that only the Z1 isoform is functional in this *Z1/4* isoform.

Effect of hydroprene application in *Tribolium*

Application of 1.5 nmoles of the JHA hydroprene to mid-sixth instar larvae caused supernumerary molts (extra larval molts after the eighth instar) in five out of 14 larvae (Table 1). Two others formed larval/pupal intermediates, with one showing mostly larval characteristics, and one with enlarged wings and intermediate larval/pupal appendages (see Fig. S1 in the supplementary material). After higher JHA doses, more became supernumerary larvae (Table 1). Most of the hydroprene-treated animals eventually died without forming a pupa. The few that subsequently molted to pupae then either died as pupae or eclosed as adults with secondary pupal cuticle (data not shown). All but one of the acetone-treated control larvae formed perfect pupae, with 3/13 molting once to a seventh (final) larval instar, then to a pupa (Table 1). The remainder molted to eighth instar larvae, then to pupae. All of these controls formed normal adults.

When freshly molted pupae (less than 4 hours after ecdysis) were treated with hydroprene, a second pupal cuticle was formed in the adult molt, the amount depending on the dose given (Fig. 2). When 0.15 nmoles hydroprene were applied, small patches of the pupal cuticle were visible on the ventral abdomen of the adult (Fig. 2, left, black arrowhead). A very small pupal patch was also visible on the midline of the pronotum (Fig. 2, right, white arrowhead). These animals eclosed properly, the wings expanded more or less normally, and the head and the wings were normally pigmented. When 1.5 nmoles hydroprene were applied, a second pupal cuticle was seen on most of the abdomen (Fig. 2, left, gray arrowhead), including pupal-specific projections called gin traps (Fig. 2, right inset, black arrow), on the pronotum (Fig. 2, right, gray arrow) and on the ventral thorax (Fig. 2, white arrows). After exposure to a 10-fold higher dose, none of the pupae ecdysed: under the first pupal cuticle, a second pupal cuticle was formed and most of the tissues were pupal, including the head (Fig. 2, left, gray arrow).

Expression of *br* isoforms in normal and JH-treated larvae

All *br* isoforms were detected at low levels by RT-PCR during the sixth instar and until around day 4 of the final seventh instar (Fig. 3A; although the signal for the *Z1/Z4* and *Z4* isoforms is not visible in Fig. 3A, an increase of two RT-PCR cycles revealed a band). On day 4 of the final instar, major increases in the amounts of mRNA were observed (Fig. 3A), and the expression remained high for the remainder of the instar. This rise in expression occurs around 1 to 2 days before the larvae enter the stationary crooked posture stage that signals the onset of visible metamorphosis (Quennedey and Quennedey, 1999). It should be noted that different numbers of cycles were used for each isoform, and, based on the number of cycles alone, *br-Z4* mRNA appears to be most abundantly expressed, whereas *br-Z2* mRNA is expressed least abundantly.

When larvae were treated with 15 nmoles hydroprene on day 2 of the sixth instar, they molted to seventh instar larvae that expressed all *br* isoforms in patterns similar to those seen in the untreated sixth instar (Fig. 3B). By contrast, those sixth instar larvae given only

Table 1. Effect of hydroprene application during the sixth instar

				Supernumerary larvae				
					Number of supernumerary molts			
	Total	Number of pupae	Number of L-P intermediates	Total number	1	2	3+	Number dead before metamorphosis
Hydroprene (150 nmol)	14	2	0	7	2	1	4	5
Hydroprene (15 nmol)	17	2	0	12	4	4	4	3
Hydroprene (1.5 nmol)	14	5	2	5	1	2	2	2
Acetone	14	13	0	0	–	–	–	1

Supernumerary larvae are larvae that underwent additional larval molts after the eighth instar. Number dead represents the number of larvae that died before forming a pupa or a larval-pupal intermediate, or before undergoing supernumerary larval molts.

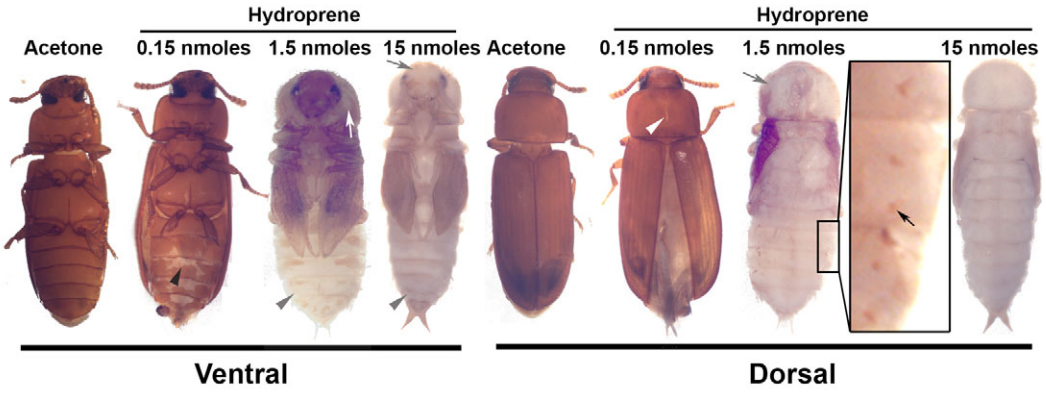

Fig. 2. Effect of application of increasing hydroprene concentration during early pupal development on the phenotype of the adults. Second pupal cuticle is visible on the pronotum (white arrow) and the abdomen (black arrowhead). At higher doses, a second pupal cuticle is also visible on the head and ventral thorax (gray arrows), and the pronotum and the ventral abdomen (gray arrowheads) have mostly pupal cuticle. (Inset) Gin traps are visible on the dorsal side (black arrow).

acetone showed *br* isoform expression profiles typical of untreated final (seventh) instars, with all isoforms dramatically increasing on day 4 (Fig. 3B).

Effect of RNA interference-mediated reduction of the *br* core expression

To assess the functional role of *br* in *Tribolium*, we injected double-stranded RNA (dsRNA) targeting the core region of *br* gene into penultimate and final instar larvae. Injection of *br* core dsRNA resulted in a reduction of the expression of each *br* isoform to trace levels during the crooked posture stage of the prepupal period when each isoform is normally expressed strongly (data not shown). There was no external morphological effect of *br* elimination during the molt from the penultimate to the last larval stage. These larvae and those receiving the dsRNA during the last instar showed a marked alteration of the pupal molt. Typically, the individual formed had a mix of larval and adult-like structures (Fig. 4), and had mobile appendages that showed rapid twitch-like movements (see Movie 1 in the supplementary material), unlike normal pupae whose appendages are immobile. In both cases, the abdomen moved when disturbed. Injection of *amp*^{r} dsRNA had no effect and resulted in the formation of normal pupae that subsequently molted to normal adults (data not shown).

The abdomen of the 'pupae' formed after injection of the *br* core-dsRNA was larval-like (Fig. 4A), with a reduced number of short setae and short adult-specific spines; it lacked the pupal-specific gin traps, and occasional brown spots were observed at their sites (Fig. 4C). The urogomphi (terminal appendages on the abdomen) were intermediate between larval and pupal in their shape and width, but showed a larval-like pigmentation (Fig. 4B,D). The dorsal abdomen had a larval-like pigmentation pattern, although it was not as dark as in the larva (Fig. 4A), and the ventral side had grooves that run anteroposterior along the lateral sides; these are conspicuous in the larva (data not shown). The sternites, however, were narrower anterioposteriorly and wider laterally, assuming a more pupal/adult-like morphology (Fig. 4A).

The appendages of the *br* core dsRNA-treated individuals had a more adult-like morphology with pronounced segmentation, and differentiation of adult type claws on the legs (Fig. 4E-I). Neither of these traits was seen in the *amp*^{r} dsRNA- or water-injected control pupae, whose appendages tended to be relatively smooth, showing only the beginnings of segmentation and no differentiation of the claws (Fig. 4E-G). The maxillae and mandibles in the *br* dsRNA-treated individuals also assumed a more adult-like morphology (Fig. 4H,I). In particular, the maxilla

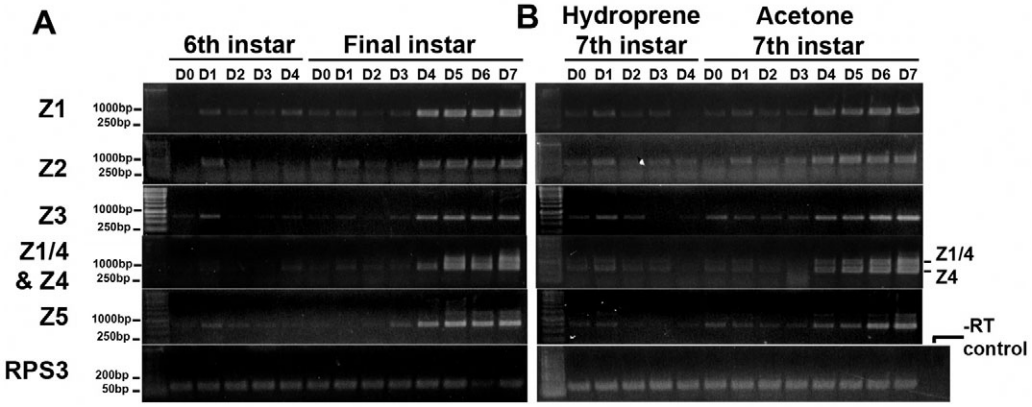

Fig. 3. Expression profiles of the *br* isoforms. (**A**) Expression profile of the *br* isoforms during development of the sixth instar and the seventh instar larvae as determined by RT-PCR. (**B**) Expression profile of the *br* isoforms during the development of the hydroprene-treated and the acetone-treated seventh instar larvae as determined by RT-PCR. The hydroprene-treated larvae molt to a supernumerary instar, and the duration of the seventh instar is truncated relative to the acetone-treated final instar larvae. Expression of *rps3* is provided to verify equal loading. Cycle numbers for *br-Z1*, *br-Z2*, *br-Z3*, *br-Z4*, *br-Z5* and *rps3* are 38, 40, 34, 32, 35 and 37, respectively. D, day.

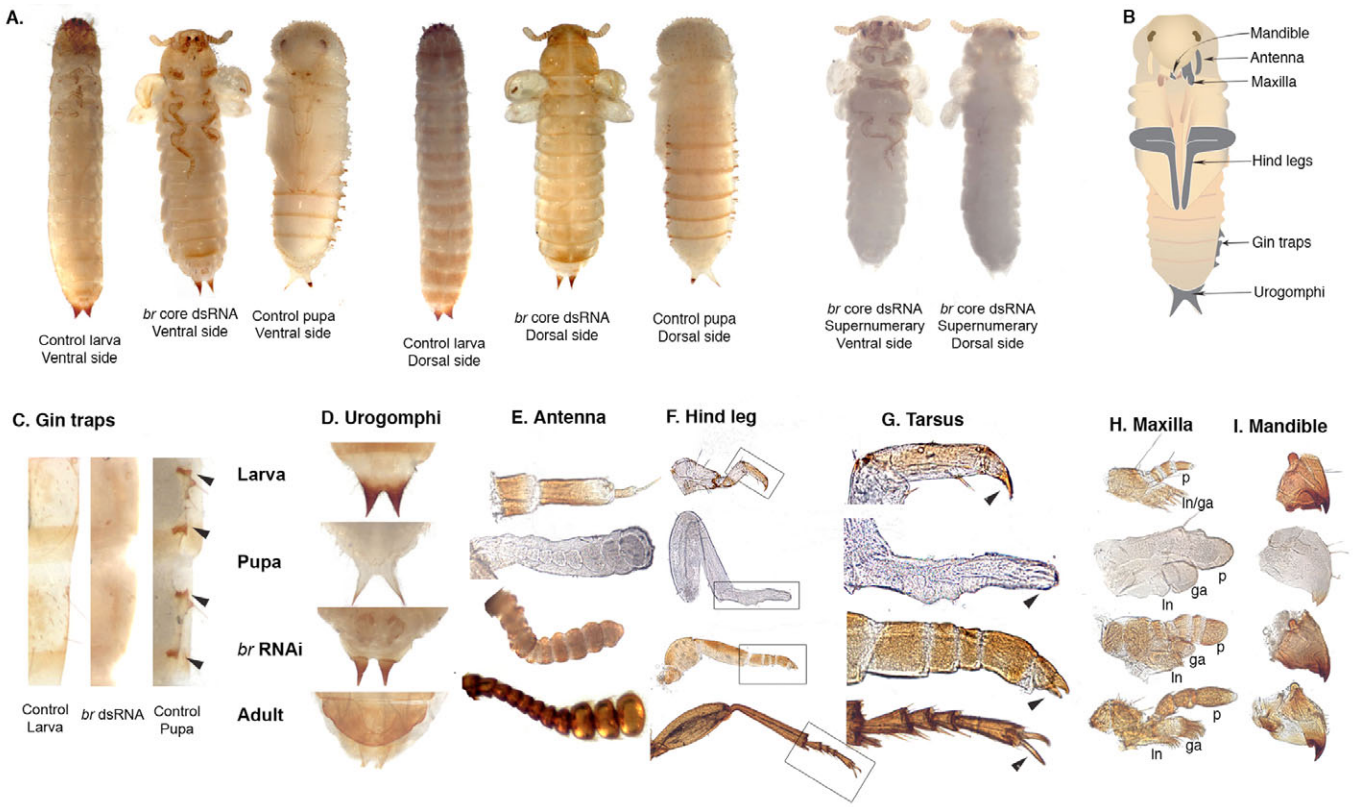

Fig. 4. Phenotypes obtained from larvae injected with *br* core dsRNA. (**A**) Ventral and dorsal views of the whole body of the larva, and the *br* dsRNA- and water-injected controls. Also shown are ventral and dorsal views of a *br* dsRNA-injected supernumerary individual, the old cuticle of which was removed. (**B**) Diagram of the body parts of *Tribolium* discussed in the text (shaded in gray). (**C**) Comparisons of the abdominal wall at the lateral side showing the presence and absence of gin traps in the untreated larva, and in the *br* dsRNA- and water-injected control animals. (**D-I**) Comparisons of the external morphology of appendages in larvae, pupae (water-injected), *br* dsRNA-injected individuals and adults. (D) The urogomphi of *br* dsRNA-injected individuals tended to appear more larva-like. (E,F) Antennae (E) and hind legs (F) of *br* dsRNA-injected individuals look more adult-like, with distinct segments and forked claws at the end of the legs. (G) Magnified view of the tarsus of the hind leg. Arrowheads indicate claws. The pigmentation of the tarsal cuticle in the *br* dsRNA-treated animals is more like that seen in the larvae. (H) The maxilla of *br* dsRNA-treated animals has an adult-like morphology with larger palps (p), and a more defined galea (ga) and lacinia (ln), but it retains the larval cuticular pigmentation pattern. (I) The morphology of the mandible of the *br* dsRNA-treated animal is most like that of the adult.

of *br* dsRNA-treated individuals had segmented palps, and well-defined lacinia and galea, both of which are not well developed in the larvae.

By contrast, the pigmentation of the tanned cuticle in these appendages resembled that of the larval appendages (Fig. 4E-G). In larval tibia, the anterior is light brown and the posterior is white (Fig. 4F,G), whereas, in adults, tibial cuticle is highly sclerotized and uniformly dark brown. The tibia of *br* dsRNA-treated individuals was pigmented similarly to the larval tibia, and the leg as a whole had larval-like sclerotization. The femur of treated legs was not expanded properly, resulting in a wrinkled and shortened femur.

Some of the individuals exposed to *br* core dsRNA (11/29, 38%) died as prepupae or during the process of ecdysis. When their old cuticle (exuviae) was removed, the animals were phenotypically similar to those that had successfully ecdysed. These larval-adult intermediates formed from larvae given *br* core dsRNA typically died within a few days. Some of them, however, underwent a molt, but not ecdysis. When we manually removed the old cuticle, the phenotype of these molted individuals was identical to that obtained after the first molt (Fig. 4A). Thus, the external morphology of these individuals was a repeat of the previous larval-adult intermediate.

Effect of isoform-specific RNA interference

Effect of a mixture of dsRNAs for all *br* isoforms

To assess which of the *br* isoforms was responsible for the observed phenotype, dsRNA for each of the isoforms was synthesized and injected into either the penultimate or final instar larvae. To determine whether suppression of all isoforms could mimic the effect of the loss of the *br* core expression, we injected larvae with a mixture of dsRNAs of all five isoforms. The resulting animals resembled the individuals obtained after giving *br* core dsRNA (compare Fig. 5B with 5C). The only difference was that the gin traps were not completely eliminated, as in those receiving the *br* core dsRNA, but they were substantially reduced in size, and tiny brown bumps were visible (Fig. 5C; arrowheads).

Effects of reductions of single isoforms

Larvae treated with either *br-Z2* or *br-Z3* dsRNA formed pupae that looked normal except for shortened wings and a minor modification of the legs, with the beginning of segmentation and weak forked claw formation (Fig. 5D,E,J,K). The overall body sizes of these pupae were not different from those of the water-injected control pupae, but the wing lengths were substantially shorter (Fig. 5D,E,J). Pupae formed after *br-Z2* dsRNA injection survived to produce a

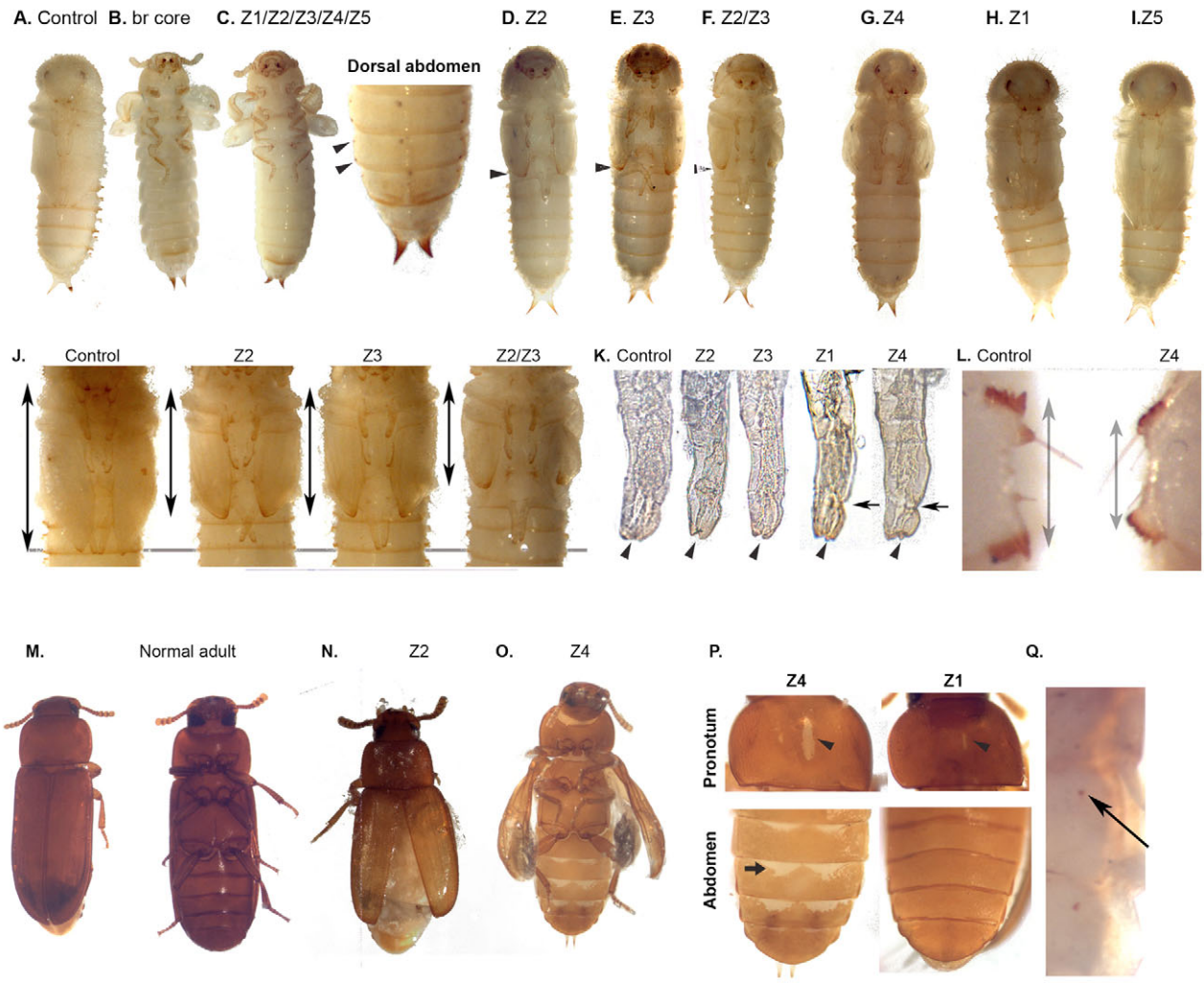

Fig. 5. Phenotypes obtained following injection of different *br* isoform dsRNA. (**A**) Water-injected control pupa. (**B**) Phenotype obtained from knockdown of the *br* core region. (**C**) Phenotype obtained from knockdown of all of the *br* isoforms. A close up of the dorsal abdomen is shown with the reduced gin traps indicated by arrowheads. (**D-F**) Phenotypes obtained from isoform-specific knockdown of *br*-Z2 (D), *br*-Z3 (E), and a combination of *br*-Z2 and *br*-Z3 (F). Arrowheads indicate the tips of the reduced wings. (**G**,**H**) Phenotypes obtained from isoform-specific elimination of *br*-Z4 (G) and *br*-Z1 (H). (**I**) Pupa injected with *br*-Z5 dsRNA looks normal. (**J**) Effect of *br*-Z2 and *br*-Z3 dsRNA injection on the wings. Gray line marks the position of the tip of the wings in the control animal. The double arrows indicate the length of wings on each pupa. (**K**) Effect of isoform-specific knockdown on the pupal tibia. Arrowheads point to the claws of the pupae; arrows indicate the slightly augmented demarcation of claw region in *br*-Z1 and *br*-Z4 pupae. (**L**) Gin traps are reduced in pupae given *br*-Z4 dsRNA. Double arrows show the length between the two gin trap projections. (**M**) Normal adult. (**N**) Adult given *br*-Z2 dsRNA. (**O**) Adult given *br*-Z4 dsRNA. (**P**) Close-up of the pupal-like cuticle on the dorsal thorax (arrowhead) and ventral abdomen (arrow) in *br*-Z1 and *br*-Z4 dsRNA-treated animals. (**Q**) A close-up of gin trap-like projections (arrow) seen on the dorsal adult abdomen in *br*-Z4 dsRNA-treated adults.

normal adult cuticle and to initiate adult ecdysis. Most, however, failed to complete ecdysis, such that the pupal cuticle remained on the tips of the wings, leading to wings that were not fully expanded. The treated adults that eclosed properly (2 out of 11) had shorter wings than those seen in normal adults, which was particularly obvious for the forewings (compare Fig. 5M with 5N). Adults from larvae given *br-Z3* dsRNA exhibited similar defects in adult eclosion.

Injection of *br-Z4* dsRNA resulted in the formation of pupae with slightly ballooned-out wings and more rounded abdomens (Fig. 5G). The legs showed a more adult-like morphology, particularly in the claw region, with the presumptive claws more defined than those seen in the control pupa (Fig. 5K; arrowhead and arrow). The gin traps were also slightly reduced in size, although the effect is subtle (Fig. 5L). Of all the animals formed from isoform-specific dsRNA-injected larvae, the morphology after exposure to *br-Z4* dsRNA was the most disrupted, although the *br-Z4* dsRNA animals still retained many of the pupal features. The adults formed after exposure to *br-Z4* dsRNA showed characteristic variably sized patches of untanned pupal-like cuticle at the anterior edge of each abdominal sternite on either side of the midline (Fig. 5O,P; arrow). A similar untanned cuticle was also seen on the midline of the pronotum (dorsal thorax; Fig. 5P; arrowhead). In addition, tiny dots were seen on the adult dorsal abdomen where pupal gin traps usually form (Fig. 5Q; arrow).

The effect of *br-Z1* dsRNA was comparatively weak. The *br-Z1* dsRNA-injected pupae formed claws that were similar to those formed following exposure to *br-Z4* dsRNA, but the wings looked

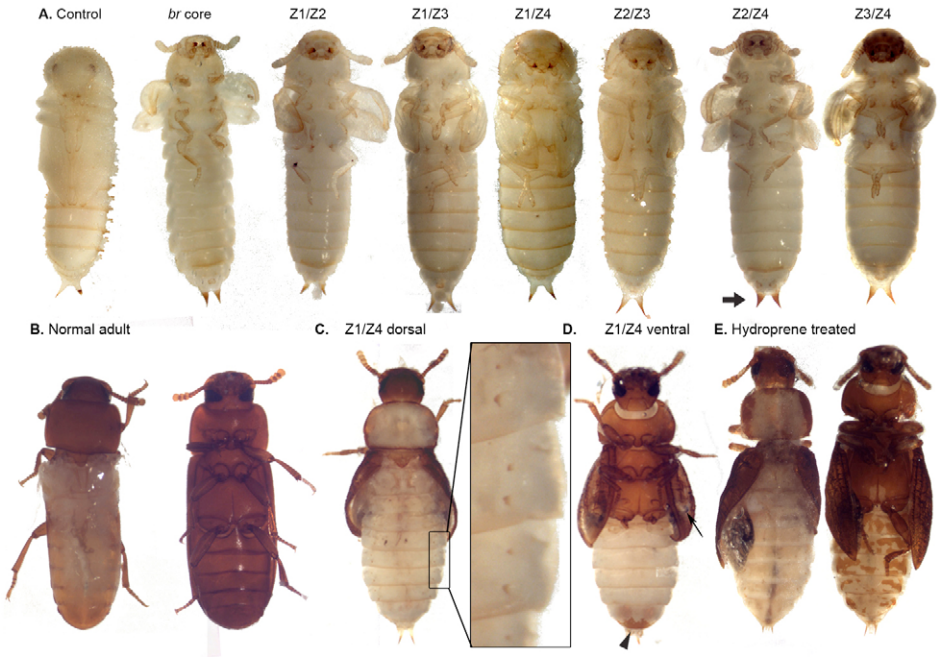

Fig. 6. Phenotypes obtained following pair-wise knockdown of *br* isoforms. (**A**) Phenotypes obtained. Arrow points to the most-affected urogomphi (*br-Z2/Z4* dsRNA-treated pupa). For comparison, pupae of a control animal and of an animal given *br* core dsRNA are shown. (**B**) Dorsal and ventral views of an untreated adult with the wings removed. (**C**,**D**) Dorsal (C) and ventral (D) sides of an adult obtained from larva given *br-Z1* and *br-Z4* dsRNA. Pupal-specific gin traps are clearly visible on the dorsal abdomen (inset). Arrowhead shows the small patch of adult cuticle at the tip of the abdomen; arrow indicates a region of wing lacking the dark brown pigmentation. (**E**) Dorsal and ventral views of an adult produced from a larva treated with 1.5 nmoles hydroprene during the prepupal stage.

more or less normal (Fig. 5H,K). Many normal-looking adults were formed after larval exposure to *br-Z1* dsRNA. A few had the stripe of untanned cuticle on the pronotum, as seen in the *br-Z4* dsRNA-treated adults, but all had proper tanning on the abdominal sternites (Fig. 5P).

br-Z5 dsRNA had no obvious phenotypic effects, and the pupae looked normal (Fig. 5I). These *br-Z5* dsRNA-treated animals survived to form normal adults.

Effects of pair-wise knock-downs

Because individuals formed after exposure to *br-Z2* or *br-Z4* dsRNA displayed features that were most similar to those seen after exposure to *br* core dsRNA, a mixture of *br-Z2* and *br-Z4* dsRNA was injected (Fig. 6A). The resulting individuals were essentially identical to the individuals that had all five isoforms reduced. Thus, a reduction of both Br-Z2 and Br-Z4 was sufficient to produce the phenotype seen after removal of all isoforms.

To assess the effect of other combination of the isoforms, pair-wise injections of *br-Z1*, *br-Z2*, *br-Z3* and *br-Z4* isoform dsRNA were performed. Injection of *br-Z1* and *br-Z2*, *br-Z1* and *br-Z3*, and *br-Z3* and *br-Z4* dsRNA all resulted in individuals that resembled the complete *br* knockdown phenotypes, except that the degrees of pigmentation and segmentation were weaker (Fig. 6A). Furthermore, the urogomphi were more pupal-like in character. Thus, these combinations produced weaker effects than those caused by giving both *br-Z2* and *br-Z4* dsRNA.

When both *br-Z2* and *br-Z3* dsRNA was injected into the same larva, a pupa with shorter wings was formed (Fig. 5F,J). The degree of shortening showed an additive effect over that caused by each of the two isoforms alone (Fig. 5J). In these animals, the relative shortening was greater for the forewing than for the hind wing.

When both *br-Z1* and *br-Z4* dsRNA was injected, the resulting pupae resembled the individuals given only *br-Z4* dsRNA, but with slightly shorter ballooning wings (compare Z1/Z4 in Fig. 6A and Z4 in Fig. 5G). These pupae subsequently molted to form adults with large patches of pupal-like cuticle on the abdomen and the pronotum (Fig. 6C,D). Only a small patch of adult cuticle was present at the very tip of the terminal abdominal segment (Fig. 6D, arrowhead). In addition, reduced gin traps were present on the adult abdomen (inset, Fig. 6C). In normal animals, gin traps are only seen on the pupal abdomen. The pronotum had mostly pupal-like cuticle, except at the margin where a small patch of adult cuticle was present (Fig. 6C). The forewings also showed patches of white pupal-like cuticle (Fig. 6D; arrowhead). In most animals, the ventral thorax and the head developed normally, but occasionally small patches of pupal-like cuticle were found. This phenotype was similar to that of the adults that were produced from animals that were treated with hydroprene during early pupal development (Fig. 6E).

Adding *br-Z5* dsRNA to either *br-Z2* or *br-Z4* dsRNA had no effect on the phenotype. The resulting animals resembled the *br-Z2* and *br-Z4* dsRNA-treated pupae, respectively (data not shown).

DISCUSSION

In the present study, we have isolated and determined the expression and function of *br* isoforms in *Tribolium*. Use of dsRNA-mediated genetic interference revealed that *br* plays a major role in directing pupal development, and that the suppression of *br* expression results in the formation of individuals with a mixture of larval and adult traits instead of a normal pupa. Similar findings are reported by Konopova and Jindra (Konopova and Jindra, 2008). We also found that the *br* isoforms interact epistatically, so that elimination of certain pairs of isoforms is necessary for the disruption of pupal development.

Evolution of *br*

All four *br* isoforms found in the more derived Lepidoptera (Zhou et al., 1998; Ijiro et al., 2004) (T. Koyama and L.M.R., unpublished), Diptera (DiBello et al., 1991; Chen et al., 2004) and Hymenoptera (Spokony and Restifo, 2007) are also found in *Tribolium*. The chromosomal isoform order of *br-Z1*, *br-Z4*, *br-Z2* and *br-Z3* is conserved among *Tribolium*, *Bombyx* and *Drosophila*. The pattern of splicing in *Tribolium* was also found to be similar to that observed in *Drosophila*, *Manduca* and *Bombyx*, with all isoforms alternatively splicing to the core region (DiBello et al., 1991; Bayer et al., 1996a; Zhou et al., 1998; Reza et al., 2004). We also identified a fifth isoform that had low amino acid similarity to *br* isoforms found in

other insects. Because our dsRNA-mediated removal of this isoform by itself or in combination with either *br*-Z2 or *br*-Z4 dsRNA had no apparent effect on the larval-pupal transition, either its function is completely redundant or it has a novel function unrelated to morphogenesis.

We found that the expression patterns of *Tribolium br* isoforms were similar to those of *Drosophila* and *Manduca* (Bayer et al., 1996a; Zhou et al., 1998; Zhou and Riddiford, 2002); they were expressed at high levels in the last larval instar but not in JH-treated supernumerary larvae. The expression of these isoforms during the prepupal period appears to correspond to the time when the ecdysteroid titer rises prior to pupation in *Tribolium* and *Tenebrio molitor* (Hirashima et al., 1995; Quennedey and Quennedey, 1999). As in *Bombyx* penultimate stage larvae (Ijiro et al., 2004; Nishita and Takiya, 2004), low levels of *br* are present earlier in *Tribolium* larvae, and even the embryos (Konopova and Jindra, 2008), but no disruption of larval development has been noted after *br* dsRNA treatment in the penultimate (our study) or earlier (Konopova and Jindra, 2008) stages. Apparently, expression of *br* isoforms during larval life does not play a major role in external larval development of Holometabola, as it does in the hemimetabolous *Oncopeltus* (Erezyilmaz et al., 2006). In both *Drosophila* and *Manduca*, *br* is expressed in certain classes of larval neurons (B. Zhou, PhD thesis, University of Washington, 2000; B. Zhou, D. Williams, J. Altman, L.M.R. and J.W.T., unpublished). This neuronal expression may represent the persistence of an ancestral nymphal function of *br* that is related to neuronal plasticity during the growth of the immature larva. Further study of these differences is warranted.

In the hemimetabolous *Oncopeltus*, *br* is expressed in all the nymphal stages during both the intermolt and the molt, except during most of the final nymphal stage and the molt to the adult (Erezyilmaz et al., 2006). Its removal by dsRNA results in a stationary molt and prevents anisomorphic growth of the wing pads. In *Tribolium*, we found that the removal of *br*, especially *br*-Z2 and *br-Z3*, resulted in pupae with shortened wings, a phenotype also seen in *Oncopeltus* adults (Erezyilmaz et al., 2006). Thus, at least the role of *br* in wing development appears to be conserved.

Tribolium lacking all Br isoforms, and those lacking only Br-Z2 and Br-Z4 isoforms, failed to make pupal structures but instead had a mix of larval and adult traits. Only when the removal of *br* was not complete or when *br* was removed much later during the prepupal period did we see some pupal traits, such as gin traps (Y.S., L.M.R. and J.W.T., unpublished). In addition, during the molt to the larval-adult intermediates, two cuticle genes were expressed that are normally expressed during the larval-larval and pupal-adult molts but not during the larval-pupal molt (Y.S., L.M.R. and J.W.T., unpublished). *br,* with Br-Z2 and Br-Z4 isoforms playing key roles, therefore, acts as a pupal specifier in *Tribolium*, as in *Manduca* and *Drosophila* (Zhou and Riddiford, 2002), leading to a specialized pupal morphology and preventing adult morphogenesis.

The effect of removal of *br* isoforms, however, differs between *Tribolium* and *Drosophila*. In *Drosophila*, *br* mutants exhibit developmental arrest at different stages of development (Kiss et al., 1988), rather than showing precocious adult development as in *Tribolium*. Removal of *br* from the silkworm *Bombyx mori* also results in disruption of metamorphosis and developmental arrest without progression into the adult morphology (Uhlirova et al., 2003). In *Bombyx*, removal of *br* results in adult legs that do not undergo proper leg morphogenesis and therefore have fewer tarsal segments. Thus, the more-derived Holometabola may have less flexibility in the sequence of life cycle stages.

Epistasis and evolutionary conservation of partial functional redundancy of *br* isoforms within holometabolous insects

Our study showed that we needed to remove pairs of *br* isoforms for the complete disruption of pupal development. Notably, removal of certain pairs of isoforms results in phenotypes that are not purely additive, which suggests an epistatic interaction between these isoforms.

The effects of the loss of isoform-specific *br* mRNA in *Tribolium* suggest that there is partial redundancy in the functions of the isoforms. Removal of either Br-Z2 or Br-Z3 reduces wing length, and pupal development is disrupted in a similar fashion when either of these isoforms is removed with either Br-Z1 or Br-Z4, suggesting that Br-Z2 and Br-Z3 have partially overlapping functions. Similarly, the pupal phenotypes resulting from the loss of Br-Z1 or Br-Z4 indicate that these two isoforms are likely to have similar functions. In *Drosophila*, Bayer et al. (Bayer et al., 1997) have found that Br-Z2 and Br-Z3, as well as Br-Z1 and Br-Z4, have partially overlapping functions during metamorphosis. Thus, these patterns of isoform overlap appear to be preserved between beetles and flies.

Based on sequence comparisons, it has been suggested that the different isoforms of *br* arose through a series of duplication events (Spokony and Restifo, 2007), with Br-Z1 and Br-Z4 having evolved most recently (Bayer et al., 1996b; Bayer et al., 2003; Spokony and Restifo, 2007). Furthermore, *Manduca* Br-Z4 has been shown to partially rescue *Drosophila* Br-Z1 functions (Bayer et al., 2003). Thus, the chromosomal arrangement, as well as the partial redundancy in the function, of these two isoforms has been maintained throughout the 300 million years that separate beetles and flies. The Br-Z1 and Br-Z4 isoforms, therefore, might have been maintained within the holometabolous insects through duplication followed by subfunctionalization (Hughes, 1994; Force et al., 1998; Spokony and Restifo, 2007) and/or functional diversification (Ohno, 1970).

Role of *br* during metamorphosis

In animals given *br* dsRNA, the larval tissues begin the transformation to adult tissues directly, but this transformation is never complete. Significant growth normally occurs during the pupal-adult transition, but these animals did not have the benefit of this extra molting period. Hence differentiation was incomplete.

We also observed that the loss of all isoforms of *br* results in the redirection of the pupal molt to form a larva-adult intermediate and that the latter then sometimes molted again to an identical larval-adult intermediate. This second molt is notable because the adult molt is typically a terminal one but the larval-adult intermediates clearly have retained an 'immature' neuroendocrine system. This 'status quo' molt is typically associated with JH (Riddiford, 1994). Thus, one possible outcome of *br* RNAi is that the prothoracic glands do not degenerate (Zhou et al., 2004) and that the corpora allata do not fully shut down as they normally do during the larval-pupal transition. Notably, the gene for *Drosophila* allatostatin, which may inhibit JH release, has several *br* isoform binding sites (Bowser and Tobe, 2007). Our preliminary experiments show that the larval nervous system does not remodel properly in animals given *br* dsRNAi. As a result, the animal might maintain an elevated larval-like JH titer in the larval-adult intermediate that induces a 'status quo' molt.

When *br-Z1* and *br-Z4* were removed in the final larval stage, the larvae first molted to fairly normal pupae (Fig. 6), presumably because *br-Z2* and *br-Z3* are still functional. They then molted to adults with patches of pupal cuticle. The reformation of pupal cuticle

during a pupal-adult molt is indicative of JH action in many holometabolous insects (for a review, see Riddiford, 1994). It also suggests a failure to shut down the JH system if the proper *br* isoforms are not expressed.

Possible role of *br* in the evolution of the holometabolous pupa

The compact form of the homolometabolous pupa is thought to have evolved from a mobile nymph-like pupa that is seen in more basal holometabolous insects, such as snakeflies (Grimaldi and Engel, 2005). It is of interest that the pupae of snakeflies have larva-like abdomens and adult-like appendages, similar to the phenotypes of *Tribolium* 'pupae' obtained from the loss of all *br*, or of selected *br*, isoforms. We hypothesize that the removal of *br* during the prepupal period in *Tribolium* recapitulates the ancestral holometabolous pupal morphology. Given the phenotypes found in the absence of all isoforms of Br at the onset of metamorphosis described here, we suggest that the evolution of the time of *br* expression (heterochrony) or tissue targets (heterotopy) of the *br* isoforms may have played an important role in the evolution of the holometabolous pupa. The evolution of Br isoform expression during the last larval stage would have led to a convergence in the development of the abdomen and the development of the imaginal primordia, leading to a specialized pupal morphology that was ecologically adaptive.

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Supplementary material

Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/135/3/569/DC1

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