Developmental profile and hormonal regulation of the transcription factors *broad* and *Krüppel homolog 1* in hemimetabolous thrips.

Chieka Minakuchi*, Miho Tanaka, Ken Miura and Toshiharu Tanaka

Graduate School of Bio-Agricultural Sciences, Nagoya University, Nagoya 464-8601, Japan.

* Corresponding author: Chieka Minakuchi, Dr. Agr.
Graduate school of Bio-Agricultural Sciences, Nagoya University, Nagoya 464-8601, Japan; Tel +81-52-789-4035; Fax +81-52-789-4032; e-mail: c_mina@agr.nagoya-u.ac.jp

Abstract

In holometabolous insects, *Krüppel homolog 1* (*Kr-h1*) and *broad* (*br*) are key players in the juvenile hormone (JH) regulation of metamorphosis: *Kr-h1* is an early JH-response gene, while *br* is a transcription factor that directs pupal development. Thrips (Thysanoptera) are classified as hemimetabolous insects that develop directly from nymph to adult, but they have quiescent and non-feeding stages called propupa and pupa. We analyzed the developmental profiles of *br* and *Kr-h1* in the western flower thrips *Frankliniella occidentalis* (Thripidae) that has one propupal instar and one pupal instar, and *Haplothrips brevitubus* (Phlaeothripidae) that has one propupal instar and two pupal instars, i.e. pupa I and pupa II. In *F. occidentalis*, the *br* mRNA levels were moderate in the embryonic stage, high at the larva-propupa transition, and low in the pre-final larval instar and the pupal stage, while *Kr-h1* mRNA levels were high in the embryonic stage, remained at a moderate level in the larval and propupal stages, and low in the pupal stage. The expression profiles in *H. brevitubus* were very similar to those in *F. occidentalis*, except that the increase of *br* expression in the final larval stage occurs more slowly in *H. brevitubus*, and that the mRNA levels of *br* and *Kr-h1* remained high in pupa I of *H. brevitubus* and then decreased. These profiles of *br* and *Kr-h1* were comparable to those in holometabolous insects, although *br* expression found in thrips' embryogenesis is reminiscent of several hemimetabolous species. Treatment with an exogenous JH mimic (JHM) in distinct developmental stages consistently resulted in lethality as pupa of *F. occidentalis* or pupa II of *H. brevitubus*. Treatment with JHM to newly molted propupae caused prolonged expression of *Kr-h1* and *br* in both species, suggesting that *Kr-h1* and *br* could be involved in mediating anti-metamorphic signals of JHM.

Keywords: juvenile hormone; thrips; metamorphosis; *broad*; *Krüppel homolog 1*

Abbreviations: *br, broad*; DMSO, dimethyl sulfoxide; JH, juvenile hormone; JHM, juvenile hormone mimic; *Kr-h1*, *Krüppel homolog 1*; *Fo*, *Frankliniella occidentalis*; *Hb*, *Haplothrips brevitubus*; *rpL32*, *ribosomal protein L32*.

1. Introduction

Insect metamorphosis is classified into three categories: ametaboly, hemimetaboly and holometaboly. The most primitive pattern is ametaboly, in which external morphology of immature individuals is a miniature form of adults, and sexual maturity is obtained as they grow through several molts. Hemimetabolous insects develop directly from the nymph to the adult, and adult structures such as wings and genitalia are formed at the end of the nymphal life. In holometabolous insects that develop from larva to pupa and then to adult, there is an apparent difference between larvae and adults in their external morphology. In this case, adult structure is rapidly formed during the quiescent pupal stage.

Insect molting and metamorphosis are regulated essentially by molting hormone (ecdysone) and juvenile hormone (JH) (Riddiford, 1994). JH was first discovered by Wigglesworth as an anti-metamorphic humoral factor in the blood-sucking bug, *Rhodnius prolixus* (Wigglesworth, 1934). Since then, the mechanisms of hormonal regulation of metamorphosis have been extensively studied in holometabolous insects such as the fruitfly *Drosophila melanogaster*, the red flour beetle *Tribolium castaneum*, the tobacco hornworm *Manduca sexta* and the silkworm *Bombyx mori* (Riddiford, 1993; Riddiford, 2008; Riddiford et al., 2003). Through the penultimate larval instar of holometabolous insects, JH is secreted continuously from the corpora allata. Ecdysone causes larval molts repeatedly in the presence of endogenous JH. After JH production ceases at the end of the larval stage, ecdysone then induces larva-pupa and pupa-adult molts, namely metamorphosis (Riddiford, 1994). Thus JH is an anti-metamorphic hormone that modifies the nature of the ecdysone-induced molts (Riddiford, 1994).

In holometabolous insects, *broad* (*br*) directs pupal development. *br* gene encodes several isoforms of transcription factors with C_2H_2-type zinc fingers that belong to the Broad-Tramtrack-Bric-a-brac (BTB) family (Bayer et al., 1996b; DiBello et al., 1991). In *D. melanogaster*, the amorphic *br* mutant can grow normally through the larval stage but cannot undergo metamorphosis (Kiss et al., 1976). *br* is necessary for the induction of pupa-specific genes (Zhou and Riddiford, 2002). RNAi-mediated knockdown studies have demonstrated that *br* is essential for pupal development in *T. castaneum* (Konopova and Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008) and *B. mori* (Uhlirova et al., 2003). In holometabolous species, *br* is expressed strongly at the larva-pupa transition, and *br* transcription is inducible by ecdysone in the absence of JH; once the level of *br* mRNA increases, however, JH works to maintain *br*

transcription (Zhou and Riddiford, 2002). Interestingly, the temporal expression pattern or the role of *br* is not consistent among Holometabola, Hemimetabola and Ametabola. In the hemimetabolous bug *Oncopeltus fasciatus*, *br* expression occurs during embryonic development and at each nymphal molt repeatedly, but then disappears in final instar nymphs; *br* promotes growth of wing pads of the pre-final instar nymphs (Erezyilmaz et al., 2006, 2009). In the ametabolous firebrat *Thermobia domestica*, *br* is constitutively expressed in the embryonic stage (Erezyilmaz et al., 2009).

In holometabolous insects, *Krüppel homolog 1* (*Kr-h1*) is a key player in JH signaling. *Kr-h1* is a transcription factor containing C_2H_2-type zinc fingers (Schuh et al., 1986) and acts as an early JH-response gene in *D. melanogaster* (Minakuchi et al., 2008) and *T. castaneum* (Minakuchi et al., 2009). During adult development of these species the endogenous JH titer is extremely low, and *Kr-h1* mRNA is absent; treatment with a JH mimic (JHM) causes up-regulation of *Kr-h1* and prevents the pupa-adult transition. Homologs of *Kr-h1* have been identified in a few holometabolous species including the honeybee *Apis mellifera* (Grozinger and Robinson, 2007), the mosquito *Aedes aegypti* (Zhu et al., 2010) and *B. mori* (Shinoda et al., unpublished). To date, detailed analysis of *Kr-h1* has not been reported in hemimetabolous species.

Thrips (Thysanoptera) belong to Hemimetabola, and are considered as the sister group of Hemiptera that includes bugs, planthoppers and aphids. The manner of thrips' metamorphosis is unusual: they undergo two or three quiescent and non-feeding stages called propupa and pupa between the larval and adult stages. As is observed in other hemimetabolous species, the external morphology of thrips larvae resembles that of adults except for the absence of adult-type wings, but there are no external wing pads in active and feeding larvae of thrips: apparently the cells in wing discs start to proliferate inside the larval cuticle at the end of the larval stage, then the wings become visible after molting to the propupa or pupa, depending on the thrips species. Larval tissues such as mouthparts and antennae partly degenerate at larva-propupa metamorphosis, and then the adult structure is formed by newly-proliferating cells. In *Frankliniella fusca* and *Haplothrips verbasci*, degeneration of the larval muscles is observed in the propupal and the pupal stages (Heming, 1973). Thus, the manner of thrips' metamorphosis is different from that of other hemimetabolous or holometabolous species.

In this study, we examined two thrips species, the western flower thrips *Frankliniella*

occidentalis (Thysanoptera, Terebrantia, Thripidae) and a predatory thrips *Haplothrips brevitubus* (Thysanoptera, Tubulifera, Phlaeothripidae), in terms of JH action. The former develops through two larval, one propupal and one pupal instars, while the latter develops through two larval, one propupal and two pupal instars (i.e. pupa I and pupa II). We show that the expression profiles of *br* and *Kr-h1* in thrips' post-embryonic development were comparable to those in holometabolous insects, while *br* expression found in thrips' embryogenesis is reminiscent of several hemimetabolous species. Treatment with an exogenous JHM in distinct developmental stages consistently resulted in lethality as pupa of *F. occidentalis* or pupa II of *H. brevitubus*. Moreover, treatment with JHM to newly molted propupae caused prolonged expression of *Kr-h1* and *br*. Our study suggests that *Kr-h1* and *br* could be involved in JH actions in thrips' metamorphosis.

2. Materials and methods

2.1. Thrips rearing

F. occidentalis were provided by Prof. T. Murai in Utsunomiya University. They were raised at 23±1ºC under a long-day photoperiod (16L-8D). Larvae and adults were fed with germinated broad bean seeds (Kokusai Pet Food, Kobe, Japan) in plastic containers according to a reported method (Murai and Loomans, 2001). Under these conditions, adult females lay eggs in the beans, and both larvae and adults feed on the beans. In case we needed more eggs, adults were put in an acrylic cylinder with tea pollen as the food source, and this cylinder was sealed with stretched Parafilm (Murai and Loomans, 2001). A few milliliters of water was put on top of the film, which was covered with a small Petri dish (36 mm in diameter). Eggs laid in water were collected on a wet filter paper every day. To obtain staged larvae, hatched larvae were collected every 24 h and reared on germinated broad bean seeds. Propupae were staged at the molt to the propupal stage, and pupae were staged at the molt to the pupal stage. In our hands, *F. occidentalis* grew up through approximately 4 days of embryonic stage, 2 days of first larval instar, 3 days of second larval instar, 1.5 days of propupal stage, and 3 days of pupal stage.

The other species, predatory *H. brevitubus*, were provided by Ishihara Sangyo Company (Osaka, Japan). They were raised with *Ephestia kuehniella* eggs (commercially available as Ento-food, from Arysta LifeScience, Japan) at 25±1ºC under a long-day photoperiod (16L-8D).

Preparation of staged animals was done as above. In our hands, *H. brevitubus* grew up through approximately 5.5 days of embryonic stage, 3.5 days of first larval instar, 5.5 days of second larval instar, 1 day of propupal stage, 1 day of pupa I stage, and 3 days of pupa II stage.

2.2. cDNA cloning

Total RNA was isolated from the whole body of *F. occidentalis* or *H. brevitubus* using a TRIzol reagent (Invitrogen, Carlsbad, CA), and the oligo-dT-primed reverse transcription was done using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara Bio, Shiga, Japan). A degenerate primer pair, br-F and br-R, was designed based on the conserved amino acid sequences in the BTB domain of other insects' Br proteins to amplify *br* from *F. occidentalis* and *H. brevitubus*. PCR with br-F and br-R primers was performed with the annealing temperature of 48ºC for *F. occidentalis*, and 40ºC for *H. brevitubus*. To obtain full-length cDNA of *Fobr*, rapid amplification of cDNA ends (RACE) was conducted as follows. Total RNA was extracted from the second-instar larvae or eggs (48-72 h after egg laying), and cDNA pools were prepared using a GeneRacer Kit (Invitrogen). Touchdown PCR and the secondary nested PCR were performed with Advantage2 Polymerase (Clontech) according to the manufacture's instructions. Some RACE-PCR reactions were performed in the presence of 5% dimethyl sulfoxide (DMSO) to suppress non-specific annealing. Primer sequences are listed in Table S1.

The cDNA cloning of *Kr-h1* from *F. occidentalis* and *H. brevitubus* was also done by using PCR-based approaches. RT-PCR with Krh1-Fa and Krh1-R1 primers was performed with the annealing temperature of 48ºC, and this was followed by the nested PCR with Krh1-Fa and Krh1-R2 primers with the annealing temperature of 46ºC. Then, the full-length cDNA sequence information of *F. occidentalis Kr-h1* was obtained by 5'- and 3'-RACE using the same cDNA pools used for *Fobr* amplification. Primer sequences are listed in Table S1. 5'- and 3'-RACE PCR was performed in the presence of 5% DMSO to suppress non-specific amplification.

The cDNA sequence of *F. occidentalis ribosomal protein L32* (*rpL32*) was obtained in a similar manner. RT-PCR with rpL32-F1 and rpL32-R1 primers was performed with the annealing temperature of 38ºC, and the nested PCR with rpL32-F2 and rpL32-R2 primers was done with the annealing temperature of 44ºC. Full-length cDNA of *ForpL32* was obtained by following 5'- and 3'-RACE. As for *H. brevitubus rpL32*, RT-PCR with rpL32-F2 and rpL32-R2 primers at the annealing temperature of 42ºC gave the partial cDNA fragment.

These PCR products were purified, cloned into pGEM-T Easy vector (Promega, Madison, WI), and sequenced. The DNA sequence data have been deposited in the GenBank under the accession numbers AB572561-AB572576 (*Fobr*), AB572582 (*Hbbr*), AB572577-AB572579 (*FoKr-h1*), AB593689 (*HbKr-h1*), AB572580 (*ForpL32*) and AB572581 (*HbrpL32*).

2.3. Quantitative RT-PCR analysis

To analyze the developmental expression profile of *F. occidentalis* genes, a bunch of staged animals were pooled for RNA isolation at each time point: 200-240 eggs for the embryonic stage, 38-67 individuals for the larval stage, and 27-31 individuals for the propupal, pupal or adult stage. We confirmed that each of the pooled animals weighed in the range of 0.7-1.0 mg. In the case of *H. brevitubus*, 70-100 eggs were pooled for the embryonic stage, 14-100 individuals for the larval stage, and 8-14 individuals for the propupal, pupal or adult stage. To analyze the transcript level in JHM-treated *F. occidentalis* or *H. brevitubus*, (see below), 8-14 individuals were pooled for RNA isolation.

Total RNA was isolated from the whole body of *F. occidentalis* or *H. brevitubus* using a TRIzol reagent with RNase-free glycogen (Invitrogen) as a carrier, treated with DNase I (Takara Bio), and reverse-transcribed using a PrimeScript 1st Strand cDNA Synthesis Kit with an oligo-dT primer.

The transcript levels of *br*, *Kr-h1* and *rpL32* were quantified using a real-time thermal cycler (Thermal Cycler Dice TP800, Takara Bio). Quantitative RT-PCR was carried out in a 16-µl reaction volume containing SYBR Premix Ex Taq (Takara Bio), 0.2 µM of each primer (see Table S1) and 1-1.5 µl of template cDNA or standard plasmids. PCR conditions were 95ºC for 30 sec, followed by 40-45 cycles at 95ºC for 5 sec and 60ºC for 30 sec. After the thermal cycling, absence of unwanted byproducts was confirmed by melting curve analysis. Serial dilutions of a plasmid containing a part of ORF of each gene were used as standards. Analysis was performed two or three times for each sample, and the average was calculated. Transcript levels of *br* and *Kr-h1* were normalized to that of *rpL32* in the same samples.

2.4. Hormonal treatments

A JHM, pyriproxyfen, was dissolved in methanol at the concentration of 3.2 µg/µl (10 mM). Staged larvae, propupae and pupae of *F. occidentalis* were anesthetized with ether for 1.5-2 min,

and were dipped into 10 mM pyriproxyfen or methanol as a control for 10 sec. After hormonal treatment, larvae were fed with germinated broad bean seeds in a small glass dish, while propupae and pupae were kept without bean seeds. A few animals that died immediately after the treatment due to the toxicity of the solvent (less than 5% of total animals tested) were excluded from the experiments. To examine the effect of JHM on the expression of *br* and *Kr-h1* in the pupal stage of *F. occidentalis*, newly molted propupae within 8 h after ecdysis were treated with 10 mM pyriproxyfen or methanol as a control, and the total RNA was extracted 48 h later. Twelve to 14 animals were pooled for RNA isolation.

Propupae and pupae (in the pupa I stage) of *H. brevitubus* were anesthetized on ice, dipped in methanol or 10 mM pyriproxyfen for 10 sec, and were reared in a small glass dish. To examine the effect of JHM on the expression of *br* and *Kr-h1*, newly molted propupae within 5 h after ecdysis were treated with 10 mM pyriproxyfen or methanol as a control, and the total RNA was extracted 72 h later. Eight to 11 animals were pooled for RNA isolation.

3. Results

3.1. cDNA sequences of *br*, *Kr-h1* and *rpL32* of *F. occidentalis* and *H. brevitubus*

Several cDNA sequences consisting of the 1044-bp core region and variable 5' and 3' sequences were obtained by RT-PCR, 5'-RACE and 3'-RACE to amplify *F. occidentalis br* (*Fobr*). Between the two 5' variable cDNA sequences of *Fobr*, the longer and the shorter variants were named as A and B isoforms, respectively (Fig. 1A). These two 5' variable sequences, probably generated by use of different promoters and alternative splicing, differ only in the 5' untranslated region, and share the same translation initiation site. Three C-terminal variable sequences encode a pair of C_2H_2 zinc finger motifs that corresponds to Z2, Z3 and Z5 isoforms reported in other insects' Brs: tblastn searches revealed that the amino acid sequence in isoform-specific regions shows the highest homology to br-Z2, Z3 and Z5 of the cockroach *Blattella germanica* (Piulachs et al., 2010). At present, cDNA sequences that correspond to Z1 and Z4 isoforms have not been amplified by 3'-RACE. Five C-terminal sequence variants lacking zinc finger motifs were also obtained (Fig. 1A). These were named as NZa (non-zinc finger a), NZb, NZc, NZd and NZe isoforms. As shown in Fig. 1B, the deduced amino acid sequence in the BTB domain on the N terminus of the core region was highly conserved with those of other insects'

Brs.

Using RT-PCR, 5'-RACE and 3'-RACE to amplify *F. occidentalis Kr-h1* (*FoKr-h1*), we obtained three distinct cDNA sequences with variable 5' regions; 2569-bp, 2835-bp and 2522-bp cDNAs were named as A, B and C isoforms (Fig. 2A). FoKr-h1 A and B isoforms, encoding a 517-aa protein, are different only in the 5' untranslated region, and share the same translation initiation site, while the amino acid sequence of FoKr-h1 C (504 aa) is only different from FoKr-h1 A or B in the N-terminal first methionine residue (Fig. 2A). Blastp analysis revealed the deduced amino acid sequence in C_2H_2-type Zn fingers was highly homologous to the Kr-h1s reported in *T. castaneum* and *D. melanogaster* (Fig. 2B). Similarly, a 461-bp cDNA encoding *ForpL32* was isolated by RT-PCR and RACE.

A 302-bp fragment for *Hbbr*, a 328-bp fragment for *HbKr-h1*, and a 191-bp fragment for *HbrpL32* were amplified by RT-PCR with degenerate primers from the cDNA pool of *H. brevitubus*. Tblastn searches showed that these are homologous to those known in other insects (91%, 87% and 92% amino acid identity with *br*, *Kr-h1* and *rpL32* of *T. castaneum*, respectively). The plasmid containing the cDNA fragment of *br*, *Kr-h1* or *rpL32* was used as the standard in quantitative RT-PCR assays.

3.2. Developmental profile of *br* and *Kr-h1* mRNA

Levels of transcript abundance were measured by quantitative RT-PCR. In *F. occidentalis*, the *Fobr* was highly expressed from Day 0 of the second larval instar (D0-L2) until Day 0 of the propupal stage (D0-ProP), and declined to a trace level by Day 1 of the pupal stage (D1-P), as shown in Fig. 3A. Importantly, *Fobr* was also expressed on Day 2 and Day 3 of the embryonic stage (D2-E and D3-E), but not abundant in the first larval stage. In *H. brevitubus*, the *Hbbr* was moderately expressed in the embryonic stage (D2&3-E and D4&5-E), remained low in the first larval instar, and gradually increased in the second larval instar (Fig. 4A). It peaked at the propupal stage, and then decreased through the beginning of pupa II.

In *F. occidentalis*, the level of *FoKr-h1* transcript was relatively high on D2-E and D3-E, and declined to much lower levels on D0-L1 (Fig. 3B). It gradually declined by D0-L2, showed a small rise on D2-L2, declined again by D1-P, and increased slightly afterwards (Fig. 3B, inset). The expression profile of *Kr-h1* in *H. brevitubus* is similar to this: the level of *HbKr-h1* transcript was high on D2&3-E and D4&5-E in the embryonic stage, moderate in the larval and propupal

stages, and undetectable in pupa II stage (Fig. 4B).

For a more accurate assessment of the reappearance of *Fobr* mRNA on D0-L2, hatched larvae of *F. occidentalis* were collected every 6 h. As shown in Fig. 3C, these *F. occidentalis* larvae begin ecdysis to the second instar (L2) at 42-48 h after hatching, and the majority have ecdysed by 60 h after hatching. The mRNA level of *Fobr* and *FoKr-h1* in the same series of samples was quantified. *Fobr* mRNA remained low until 42 h after hatching, and then increased rapidly as the ratio of the second instar larvae increased (Fig. 3C). Thus, the timing when the *br* transcript level increased in the larval stage was different between *F. occidentalis* and *H. brevitubus*: *br* mRNA increased rapidly just after L2 molt in *F. occidentalis* (Figs. 3A and 3C), while *br* mRNA increased gradually in the second half of the second larval instar in *H. brevitubus* (Fig. 4A). The transcript level of *FoKr-h1* was high until 36 h after hatching, and gradually decreased thereafter, while the transcript level of *HbKr-h1* gradually declined from D3-L1 to D2-L2 (Fig. 4B). Thus, an increase in *br* mRNA and a decrease in *Kr-h1* mRNA occur at the beginning of the final larval instar in both thrips species, but apparently it progresses more rapidly in *F. occidentalis* compared with *H. brevitubus*.

3.3. Effects of JHM treatment on thrips metamorphosis

The effect of pyriproxyfen, a JHM, on growth and metamorphosis of *F. occidentalis* was examined at various developmental stages. As shown in Table 1, treatment with JHM in the larval stage resulted in severe lethality especially in pupae: the majority of JHM-treated larvae died in the pupal stage, while 55-84% of solvent-treated controls eclosed normally. Three to 14% of animals treated with JHM in the larval stage molted to adults but died within 3 days after eclosion. We noticed that ecdysis to the adult was incomplete in some JHM-treated animals, in which a part of exuviae was still stuck on the tip of the wings or the abdomen. In some cases it was hard to identify from morphological observations at which stage animals died because they dehydrated quickly. Treatment with JHM caused lethality in the larval stage in 11-22% animals, but these values were comparable to those found in the solvent-treated controls (0-26%, Table 1). Supernumerary larval molting after JHM treatment was not observed.

Similar experiments with animals in late stages were also done. Treatment with JHM in the propupal stage at 0-18 h after ecdysis mainly resulted in lethality in the pupal stage (91%), while 97% of the controls molted to normal adults (Table 2). Treatment with JHM in the 18-36

h propupal stage resulted in lethality in the propupal (26%) or the pupal stage (50%), while 85% of the controls molted to normal adults. Then, the effect of JHM treatment to the pupa was examined (Table 3). Treatment of JHM at 0-24 h after pupal ecdysis caused lethality as pupae (66%) or newly-eclosed adults (14%), while the majority of the controls (97%) molted to normal adults; viable adults with deformed wings were also observed after JHM treatment (20%). Treatment of JHM at 24-48 h after pupal ecdysis caused lethality as pupae (95%) or newly-eclosed adults (5%), while 65% of the controls molted to normal adults. With treatment of JHM at 48-72 h after pupal ecdysis, 72% animals died as pupae, and the rest molted to normal adults, while 74% of the controls molted to normal adults. Thus, JHM treatment at 0-24 h or 24-48 h after pupal ecdysis caused severe lethality, and this effect was weaker in the animals with JHM treatment at 48-72 h after pupal ecdysis. Treatment of the solvent at 24-48 h or 48-72 h after pupal ecdysis also caused relatively high lethality as pupae (35% and 26%), probably because they were very sensitive to the toxicity of methanol at this stage. Taken together, treatment with pyriproxyfen in distinct developmental stages of *F. occidentalis* consistently resulted in lethality as pupa.

We also examined the effect of JHM treatment on *H. brevitubus* metamorphosis. As shown in Table 4, treatment of JHM in the propupal stage resulted in lethality mainly (62%) in the pupa II stage. Treatment of JHM in the pupa I stage also significantly (97%) prevented the pupa II-adult transition.

3.4. Effect of JHM on the expression of *br* and *Kr-h1*

As shown in the previous sections, treating *F. occidentalis* with JHM in the larval, propupal, or early pupal stage resulted in significant lethality in the pupal stage (Tables 1-3). As shown in Fig. 3A, the level of *Fobr* mRNA was the highest on D2-L2, declined in the propupal stage, and remained low in the pupal stage. To examine the hormonal regulation of *Fobr* in the pupal stage, newly molted propupae within 8 h after ecdysis were treated with JHM or solvent, and the transcript level of *Fobr* was measured 48 h later. At 48 h after treatment, all (14/14) solvent-treated propupae had molted to viable pupae, while 12 out of 15 JHM-treated propupae molted to pupae, and the rest (3/15) died as propupae. These dead propupae were not included for further RNA isolation or quantitative RT-PCR. Quantitative RT-PCR showed that the level of the *Fobr* transcript in control animals declined to 19% of that in propupae at 0-8 h after

ecdysis (Fig. 5A). By contrast, *Fobr* mRNA level in JHM-treated pupae stayed about 5 times as high as that in solvent-treated controls, which is comparable to the level in 0-8 h propupae (Fig. 5A).

The level of the *FoKr-h1* transcript in the same samples was also measured (Fig. 5B). At 48 h after solvent treatment, the level of the *FoKr-h1* transcript was 11% of that in propupae at 0-8 h after ecdysis. By contrast, treatment with JHM brought about maintenance of *FoKr-h1* mRNA level, being up to 8 times over that of solvent-treated controls.

We also treated newly molted propupae of *H. brevitubus* with JHM or solvent, and measured the transcript level of *br* and *Kr-h1* at 72 h later. As shown in Fig. 5C, the level of the *Hbbr* transcript in control animals was 5% of that in propupae at 0-5 h after ecdysis, but *Hbbr* mRNA level in JHM-treated pupae remained high (Fig. 5C). The level of the *HbKr-h1* transcript in the same samples was also measured (Fig. 5D). At 72 h after solvent treatment, the level of the *HbKr-h1* transcript was 1% of that in propupae at 0-5 h after ecdysis, whereas treatment with JHM caused prolonged expression of *HbKr-h1* mRNA.

4. Discussion

Thrips undergo an unusual manner of metamorphosis, and the hormonal background is yet to be determined. In this study, we presented the developmental expression profiles of *Kr-h1* and *br* and data concerning the possible roles of *Kr-h1* and *br* in JH signaling in two species of thrips. JH regulation of insect metamorphosis via *br* has been reported in holometabolous species such as *D. melanogaster* (Zhou and Riddiford, 2002), *B. mori* (Reza et al., 2004), *M. sexta* (Zhou and Riddiford, 2002) and *T. castaneum* (Konopova and Jindra, 2008; Minakuchi et al., 2009; Suzuki et al., 2008), and in hemimetabolous *O. fasciatus* (Erezyilmaz et al., 2006). *Kr-h1* has been reported as the key mediator of JH signals in *D. melanogaster* and *T. castaneum* (Minakuchi et al., 2009; Minakuchi et al., 2008).

This is the first report on the cDNA sequence and the developmental profile of *Kr-h1* in a hemimetabolous species. The level of the *Kr-h1* transcript in thrips was high in embryogenesis, moderate in the larval and propupal stages, and extremely low in the pupal stage (or the pupa II stage of *H. brevitubus*), which is comparable to those reported in *D. melanogaster* (Minakuchi et al., 2008; Pecasse et al., 2000) and *T. castaneum* (Minakuchi et al., 2009). In *T. castaneum*,

continuous *Kr-h1* expression is necessary for the maintenance of the larval status (Minakuchi et al., 2009). In *D. melanogaster* and *T. castaneum*, anti-metamorphic signaling of exogenous JHM to prevent adult development is mediated by prolonged expression of *Kr-h1* and *br* (Konopova and Jindra, 2008; Minakuchi et al., 2009; Minakuchi et al., 2008; Zhou and Riddiford, 2002). This study has demonstrated that the expression of *Kr-h1* and *br* could be prolonged into the pupal stage of thrips by giving exogenous JHM to the propupa (Fig. 5), raising a possibility that *Kr-h1* and *br* could be involved in mediating JH signals in thrips. Interestingly, the level of Kr-h1 in thrips' embryos is relatively high (Figs. 3B and 4B). It has been suggested that *Kr-h1* is essential for embryonic development of *D. melanogaster*, since a mutant line that lacks the β isoform of *Kr-h1* is lethal mainly in the embryonic stage (Beck et al., 2004). High embryonic expression of *Kr-h1* in thrips might be crucial for embryonic development.

We examined the developmental profile of *br* mRNA in two thrips species, *F. occidentalis* and *H. brevitubus*. In both thrips species, the level of *br* mRNA was low in the first larval instar, and high at the larva-propupa transition, which correlates with the onset of development of adult structures such as adult antennae and wings, as illustrated in Fig. 6B. The timing of the increase in the *br* transcript differed slightly between *F. occidentalis* and *H. brevitubus*: the increase of *br* mRNA occurred more rapidly in *F. occidentalis* compared with *H. brevitubus* (Figs. 3A, 3C and 4A). It would be interesting to compare the mechanism of hormonal regulation of *br* induction between these two species. In both thrips species, *br* expression was also observed in the latter half of the embryonic stage (Figs. 3A and 4A).

It is of great interest to know how the manner of thrips' metamorphosis has evolved. According to the fossil records, it is likely that holometabolous insects emerged from hemimetabolous ancestors. It has been proposed that the larval instars of Holometabola arose from the hemimetabolous pronymph, i.e. the final stage of embryonic development with abundant JH (Truman and Riddiford, 1999; Truman and Riddiford, 2002), and that the JH titer and *br* expression has shifted in the course of the evolution of holometabolous metamorphosis (Erezyilmaz et al., 2006). Here we compare the expression profile of *br* and wing development in hemimetabolous hemipteran bugs, *O. fasciatus*, two hemimetabolous thrips species, *F. occidentalis* and *H. brevitubus*, and holometabolous coleopteran beetles, *T. castaneum* (Fig. 6). The expression of *br* in the thrips' embryos was comparable to that of other hemipteran species such as *O. fasciatus* (Erezyilmaz et al., 2009) (Fig. 6A) and the cockroach *B. germanica*

(Piulachs et al., 2010). In the holometabolous *T. castaneum* (Fig. 6C) and *D. melanogaster*, the level of *br* mRNA is low in the embryos (Konopova and Jindra, 2008; Minakuchi et al., 2009; Suzuki et al., 2008). *br* expression in *D. melanogaster* embryos is confined to the central nervous system (Zhou et al., 2009), and the amorphic *br* mutant can grow normally through the larval stages but cannot undergo metamorphosis (Kiss et al., 1976), suggesting that *br* is not essential for embryonic development. In contrast, the expression profile of *br* in the post-embryonic development of thrips (Fig. 6B) is more similar to that of holometabolous species than to that of Hemiptera. In holometabolous *T. castaneum* (Fig. 6C), *br* expression remains low in the pre-final larval stage, then becomes abundant at the larva-pupa transition and is necessary for normal wing development (Konopova and Jindra, 2008; Minakuchi et al., 2009; Suzuki et al., 2008). Similar profiles of *br* expression have been reported in other holometabolous species (Andres et al., 1993; Ijiro et al., 2004; Nishita and Takiya, 2004; Reza et al., 2004; Zhou et al., 1998; Zhou and Riddiford, 2002). In the hemimetabolous milkweed bug *O. fasciatus*, by contrast, *br* mRNA is maintained at each nymphal molt and is needed for the progressive growth of wing pads, then disappears at the nymph-adult transition (Fig. 6A) (Erezyilmaz et al., 2006). We suppose that feeding larval instars might have arisen in ancestral thrips, and that the subsequent nymphal stages might have become non-feeding periods, which are now designated as the propupal or pupal stages. Similarity in the developmental expression profile of *br* in post-embryonic development between Thysanoptera and Holometabola (see Fig. 6) supports the idea that the larval stage of thrips corresponds to the larval stage of Holometabola, although the metamorphosis of Thysanoptera may have evolved independently from that of Holometabola.

Thysanoptera are divided into Terebrantia suborder and Tubulifera suborder, but it is not clear which suborder is more highly evolved. From morphological and histological observation, Heming (1973) suggested that an additional pupa-like instar of Tubulifera arose between the propupal and pupal instars of Terebrantia, and therefore Tubulifera is more highly evolved than Terebrantia: in other words, the propupa of Terebrantia is equivalent to the propupa and pupa I of Tubulifera, and the pupa of Terebrantia is equivalent to the pupa II of Tubulifera (Heming, 1973). Comparison of the developmental profile of *br* and *Kr-h1* as well as the effect of exogenous JHM treatment between *F. occidentalis* (Terebrantia, Thripidae) and *H. brevitubus* (Tubulifera, Phlaeothripidae) indicated that the pupa of Terebrantia might correspond to the pupa II of

Tubulifera (Figs. 3, 4 and 6). This period may also correspond to the pupa of Holometabola and the final nymphal instar of Hemimetabola, in which adult development progresses in the absence of JH and the Br protein.

Since injection or feeding of double-stranded RNA to *F. occidentalis* larvae so far has not been effective in knocking down expression of a particular mRNA (data not shown), at present we cannot examine the function of each isoform of *br* or *Kr-h1*. In *D. melanogaster*, *br* isoforms are expressed in a tissue-specific and stage-specific manner, and each isoform has different functions (Bayer et al., 1996a; Zhou and Riddiford, 2002). In *T. castaneum*, RNAi-mediated knockdown experiments have suggested that each *br* isoform has different functions (Suzuki et al., 2008). In *D. melanogaster*, three isoforms of *Kr-h1* are expressed in a stage-specific manner, and each has a different role (Pecasse et al., 2000).

To elucidate the manner of JH regulation in more detail, measurement of endogenous JH level by analytical methods would be needed. The molecular types of JH in thrips have not yet been identified. JH-0, JH-I and JH-II have been found in lepidopteran insects (Riddiford, 1994; Schooley et al., 1984), and most other insects utilize JH-III, with some exceptions such as JH bisepoxide in higher Diptera (Richard et al., 1989) or JH skipped bisepoxide in the hemipteran bug *Plautia stali* (Kotaki et al., 2009). Although we have been trying to quantify JH using homogenized *F. occidentalis* larvae (up to 16 mg) using LC-MS, the attempts have been unsuccessful so far; a larger amount of thrips would definitely be necessary to detect JH (K. Furuta, C. Minakuchi and T. Shinoda, unpublished). Therefore, in this study, the effects of exogenously-given JHM at each developmental stage were examined. In *F. occidentalis*, it is possible that the endogenous JH is not abundant in the pupa, since an exogenous JHM treatment mainly resulted in lethality as pupae despite the stage of JHM treatment (Tables 1-3), while endogenous JH might not be abundant in the pupa II stage in *H. brevitubus* (Table 4). We assume that pyriproxyfen, a JHM used in this study, is quite stable in thrips, and that the persisting residual JHM prevents the pupa-adult transition.

Taken together, our data suggested that *br* expression coincides with propupal and pupal development of thrips, and that *Kr-h1* and *br*, the key mediators of JH signals in Holometabola, could be involved in JH actions in thrips' metamorphosis. Some thrips species including *F. occidentalis* are serious agricultural pests around the world. Further studies such as analyzing chemical structure of the endogenous JH and identification of the JH receptor protein should help

in developing highly selective insect growth regulators that inhibit thrips' metamorphosis.

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Figure captions

Figure 1. Structure of *F. occidentalis br*.

(A) Schematic representation of the cDNA structure of *Fobr*. The sequence data have been deposited in the Genbank under the accession numbers AB572561 (A-Z2 isoform), AB572562 (A-Z3 isoform), AB572563 (A-Z5 isoform), AB572564 (A-NZa isoform), AB572565 (A-NZb isoform), AB572566 (A-NZc isoform), AB572567 (A-NZd isoform), AB572568 (A-NZe isoform), AB572569 (B-Z2 isoform), AB572570 (B-Z3 isoform), AB572571 (B-Z5 isoform), AB572572 (B-NZa isoform), AB572573 (B-NZb isoform), AB572574 (B-NZc isoform), AB572575 (B-NZd isoform), and AB572576 (B-NZe isoform). (B) Alignment of protein sequences of Br BTB domains. Fo, *F. occidentalis*; Ad, *Acheta domesticus* (GenBank accession number: ABA02190); Of, *O. fasciatus* (ABA02191); Tc, *T. castaneum* (NP_001104734); Bm, *B. mori* (NP_001036976); Dm, *D. melanogaster* (NP_726752). Amino acid residues shared by four or five species are indicated by shaded letters, and those common in six species are shown in white letters over black background.

Figure 2. Structure of *F. occidentalis Kr-h1*.

(A) Schematic representation of the cDNA structure of *FoKr-h1*. The sequence data have been deposited in the Genbank under the accession numbers AB572577 (A isoform), AB572578 (B isoform), and AB572579 (C isoform). (B) Alignment of protein sequences of the Kr-h1 zinc-finger domain. Fo, *F. occidentalis*; Tc, *T. castaneum* (NP_001129235); Dm, *D. melanogaster* (NP_477467). Amino acid residues identical in two species are shaded, and those shared by all three species are shown in white letters over black background. Eight putative zinc-finger motifs are indicated with red lines.

Figure 3. Developmental expression profiles of *F. occidentalis br* and *Kr-h1*.

Transcript levels of *Fobr* and *FoKr-h1* were determined by quantitative RT-PCR, and the values were normalized to those of *ForpL32*. (A) Transcript levels of *Fobr*. (B) Transcript levels of *FoKr-h1*. Inset of (B) shows the levels of *FoKr-h1* in post-embryonic development by enlarging the Y-axis. In (A) and (B), total RNA was isolated from a pool of individuals for each time point. Numbers on the abscissa indicate the ages in days. The highest values during

development (i.e. D2-L2 for *Fobr*, and D2-E for *FoKr-h1*) were set to 100% for respective genes. (C) Transcript levels of *Fobr* (solid circles) and *FoKr-h1* (open circles) around the L1-L2 transition. For more accurate staging, hatched larvae were collected every 6 h, and the RNA was isolated from a pool of individuals (N=24-33). The highest values during this period were set to 100% for respective genes. Solid squares with dashed line represent the ratio of larvae that have already entered L2 in each sample.

Figure 4. Developmental expression profiles of *H. brevitubus br* and *Kr-h1*.

Transcript levels of *Hbbr* (A) and *HbKr-h1* (B) were determined by quantitative RT-PCR, and the values were normalized to those of *HbrpL32*. Numbers on the abscissa indicate the ages in days. Total RNA was isolated from a pool of individuals. The value at the propupal stage was set to 100% for respective genes.

Figure 5. Transcript levels of *br* and *Kr-h1* after JHM treatment.

(A, B) Newly molted propupae of *F. occidentalis* within 8 h after ecdysis were treated with JHM (10 mM pyriproxyfen) or solvent only, and the *Fobr* (A) and *FoKr-h1* (B) transcripts were quantified 48 h later. Pools of 12 to 14 animals were used for RNA isolation. The values were normalized to those of *ForpL32*. The transcript levels in 0-8 h-old propupae were set to 100% for respective genes. (C, D) Newly molted propupae of *H. brevitubus* within 5 h after ecdysis were treated with JHM (10 mM pyriproxyfen) or solvent only, and the *Hbbr* (C) and *HbKr-h1* (D) transcripts were quantified 72 h later. Pools of 8 to 11 animals were used for RNA isolation. The values were normalized to those of *HbrpL32*. The transcript levels in 0-5 h-old propupae were set to 100%.

Figure 6. Diagram of expression profile of *br* in hemimetabolous Hemiptera and Thysanoptera, and holometabolous Coleoptera.

Gray bars show the developmental stages when *br* expression is present in the three insect orders: (A) hemimetabolous hemipteran bugs, *Oncopeltus fasciatus* (Erezyilmaz et al., 2006; Erezyilmaz et al., 2009), (B) hemimetabolous thrips, *F. occidentalis* and *H. brevitubus*, and (C) holometabolous coleopteran beetles, *Tribolium castaneum* (Konopova and Jindra, 2008; Minakuchi et al., 2009; Suzuki et al., 2008). Developing wings or wing pads are marked in blue.

In these three orders, high *br* expression in post-embryonic development correlates with the onset of wing development.

Table(s)

Table 1. Effect of pyriproxyfen treatment on metamorphosis of *F. occidentalis* larvae

Hormonal treatment			No. of individuals died in each stage (%)					Viable adults
Stage (h after hatching)	Compound	N	Larva	Propupa	Propupa or pupa	Pupa	Newly-ecdysed adult	
D0-L1 (0-24h)	solvent	25	2 (8)	1 (4)	0 (0)	1 (4)	0 (0)	21 (84)
	JHM	28	3 (11)	2 (7)	2 (7)	15 (54)	4 (14)	2 (7)
D1-L1 (24-48h)	solvent	31	5 (16)	0 (0)	0 (0)	0 (0)	0 (0)	26 (84)
	JHM	23	4 (17)	0 (0)	2 (9)	14 (61)	2 (9)	1 (4)
D0-L2 (48-72h)	solvent	30	2 (7)	1 (3)	3 (10)	0 (0)	0 (0)	24 (80)
	JHM	36	8 (22)	4 (11)	8 (22)	14 (39)	1 (3)	1 (3)
D1-L2 (72-96h)	solvent	27	7 (26)	1 (4)	0 (0)	4 (15)	0 (0)	15 (55)
	JHM	34	6 (18)	2 (6)	0 (0)	22 (65)	2 (6)	2 (6)
D2-L2 (96-120h)	solvent	28	0 (0)	0 (0)	0 (0)	8 (29)	2 (7)	18 (64)
	JHM	30	4 (13)	4 (13)	0 (0)	21 (70)	1 (4)	0 (0)

Larvae were dipped into JHM solution (10 mM pyriproxyfen in methanol) or the solvent.

Table(s)

Table 2. Effect of pyriproxyfen treatment on metamorphosis of *F. occidentalis* propupae

Hormonal treatment			No. of individuals died in each stage (%)				Viable adults
Stage (h after molting to propupa)	Compound	N	Propupa	Propupa or pupa	Pupa	Newly-ecdysed adult	
0-18h	solvent	34	0 (0)	0 (0)	1 (3)	0 (0)	33 (97)
	JHM	35	2 (6)	0 (0)	32 (91)	1 (3)	0 (0)
18-36h	solvent	32	0 (0)	3 (9)	0 (0)	2 (6)	27 (85)
	JHM	26	7 (26)	0 (0)	13 (50)	3 (12)	3 (12)

Propupae were dipped into JHM solution (10 mM pyriproxyfen in methanol) or the solvent.

Table(s)

Table 3. Effect of pyriproxyfen treatment on metamorphosis of *F. occidentalis* pupae

Hormonal treatment			No. of individuals died in each stage (%)		Viable adults (deformed wings)	Normal adults
Stage (h after molting to pupa)	Compound	N	Pupa	Newly-ecdysed adult		
D0-P (0-24h)	solvent	34	1 (3)	0 (0)	0 (0)	33 (97)
	JHM	35	23 (66)	5 (14)	7 (20)	0 (0)
D1-P (24-48h)	solvent	20	7 (35)	0 (0)	0 (0)	13 (65)
	JHM	20	19 (95)	1 (5)	0 (0)	0 (0)
D2-P (48-72h)	solvent	19	5 (26)	0 (0)	0 (0)	14 (74)
	JHM	18	13 (72)	0 (0)	0 (0)	5 (28)

Pupae were dipped into JHM solution (10 mM pyriproxyfen solution) or the solvent.

Table4

Table 4. Effect of pyriproxyfen treatment on metamorphosis of *H. brevitubus* propupae and pupae I

Hormonal treatment			No. of individuals died in each stage (%)				Normal adults
Stage	Compound	N	Propupa	Pupa I	Pupa II	Newly-ecdysed adult	
propupa	solvent	27	1 (4)	0 (0)	1 (4)	1 (4)	24 (89)
	JHM	29	5 (17)	5 (17)	18 (62)	1 (4)	0 (0)
pupa I	solvent	22	–	1 (5)	6 (27)	1 (5)	14 (63)
	JHM	31	–	0 (0)	30 (97)	1 (3)	0 (0)

Propupae or pupae were dipped into JHM solution (10 mM pyriproxyfen solution) or the solvent.

Table S1. Oligonucleotide primers used for RT-PCR and quantitative RT-PCR.

Primers	Sequences (5' – 3')
RT-PCR	
br-F	YTNMGNTGGAAYAAYTAYCA
br-R	ACYTCNGCNGTYTTNARRAA
Krh1-Fa	GNMGNATHCAYACNAARGA
Krh1-R1	TGNARYTTNARNACRTGRTT
Krh1-R2 (nest)	TTNARNACRTGRTTRTANCCRAA
rpL32-F1	ATHRTNAARAARMGNACNAA
rpL32-F2 (nest)	MGNCAYCARWSNGAYMGNTA
rpL32-R1	TTYTGCATNADNARNAYYTC
rpL32-R2 (nest)	ARYTCNYKNACRTTRTGNAC
5'-RACE	
Fobr-RR1	ACTACCCGGTGGGCTTTCAGGCTCTT
Fobr-RR2 (nest)	TTCAGGCTCTTCCCATCACAGGCAAT
FoKrh1-RR0	CTGCTTGGAGCAGGTGAAGCCCTTG
FoKrh1-RR1 (nest)	GCTCTGGATGAACGTCTTGCTGCAGAT
FoKrh1-RR2 (nest)	CGCCCGCAGATTTCACATTTGTACGG
ForpL32-RR1	GGGCATCAGGTACTGGCCCTTGAAAC
3'-RACE	
Fobr-RF1	TCTGCCTGCAGCCCTTACTTTCGTGA
Fobr-RF3	ATGGGACCGAGAACGAGAGCGTGAT
Fobr-RF4 (nest)	GAATGGGAGCGAGAAGACCGCCTTT
FoKrh1-RFa	CAAGGAGCGGCCGTACAAATGTGAAA
FoKrh1-RF1	AAGATCTGCGGCAAGGGCTTCACCT
FoKrh1-RF2 (nest)	CTCCAAGCAGCTCAAGGTGCACAACC
ForpL32-RF1	CGGTTATGGAAGCAATGCCAAGACCA
Quantitative PCR	
Fobr-QF1	GACTATTGCCTGTGATGGGAAGA
Fobr-QR1	AGTCAGCAAAAGCCACATCTTGT
FoKrh1-QF1	ACATCTGCAGCAAGACGTTCATC
FoKrh1-QR1	GTTGTGCACCTTGAGCTGCTT
ForpL32-QF1	CTGGCGTAAACCTAAGGGTATTGA
ForpL32-QR1	AAGCACCTTCTTGAACCCAGTC
Hbbr-QF1	AGTTTAAAGGCACATCGGGTTG
Hbbr-QR1	CAAACGCAACATCCTGAAGAAC
HbKrh1-QF1	TCCGAGTGCAACAAGACTTTCA
HbKrh1-QR1	TTGGAACAAGTAAAGCCCTTGC
HbrpL32-QF1	TGGAGGAAGCCTAAGGGTATTG
HbrpL32-QR1	GAAACCAGTGGGTAACATGTGC

Figure1

Fig. 1.

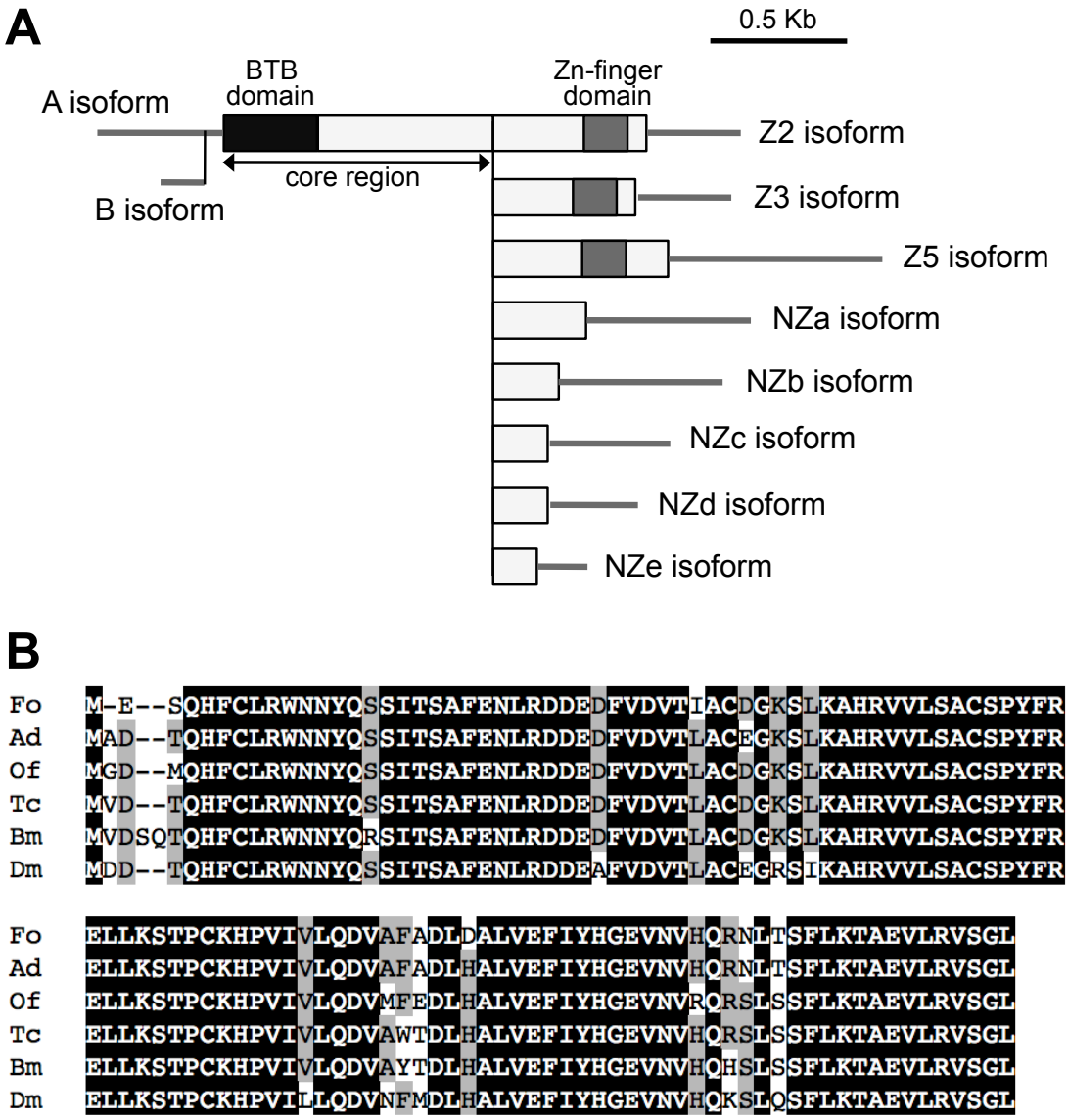


Figure2

Fig. 2.

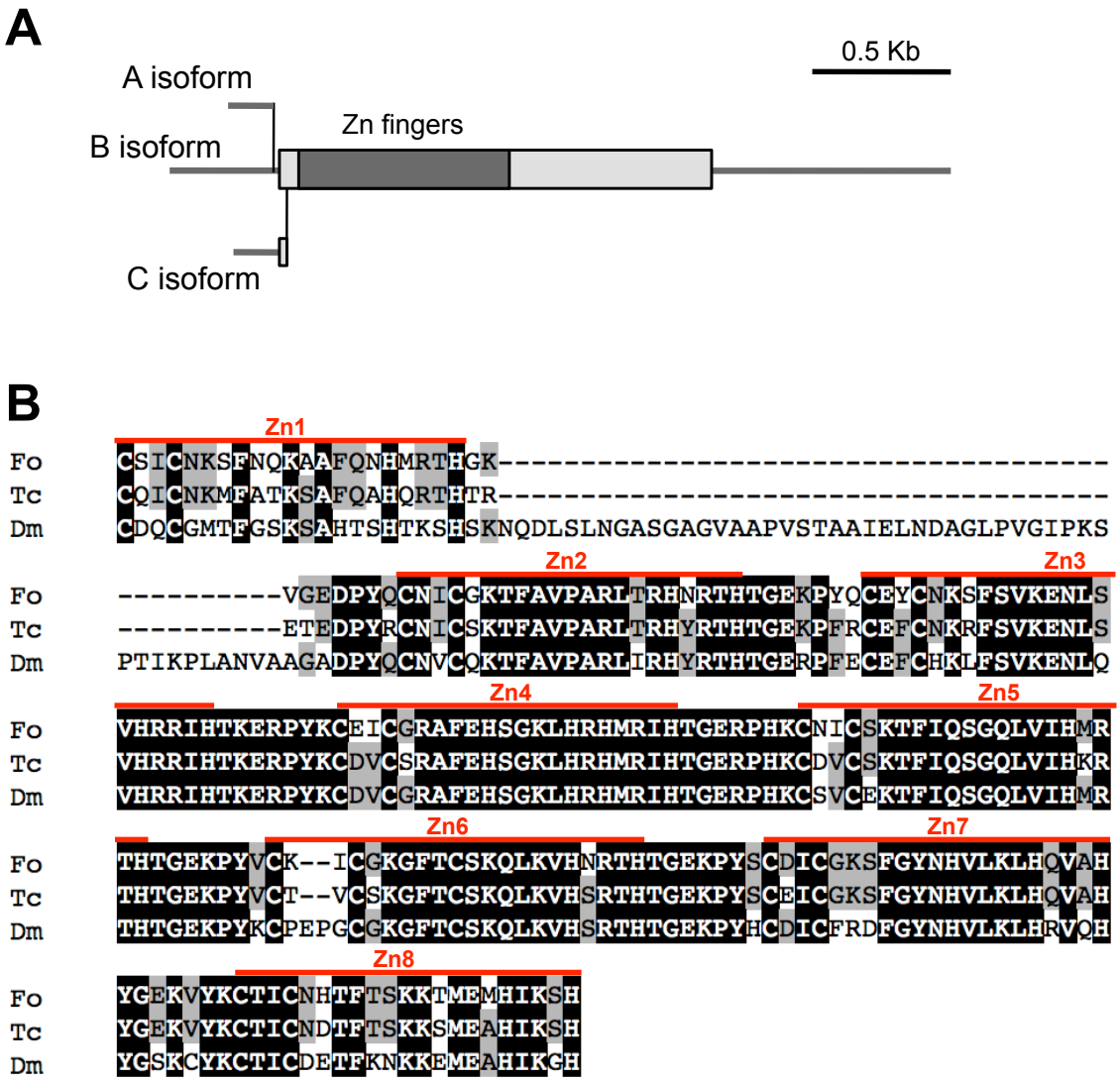


Figure3AB

Fig. 3.

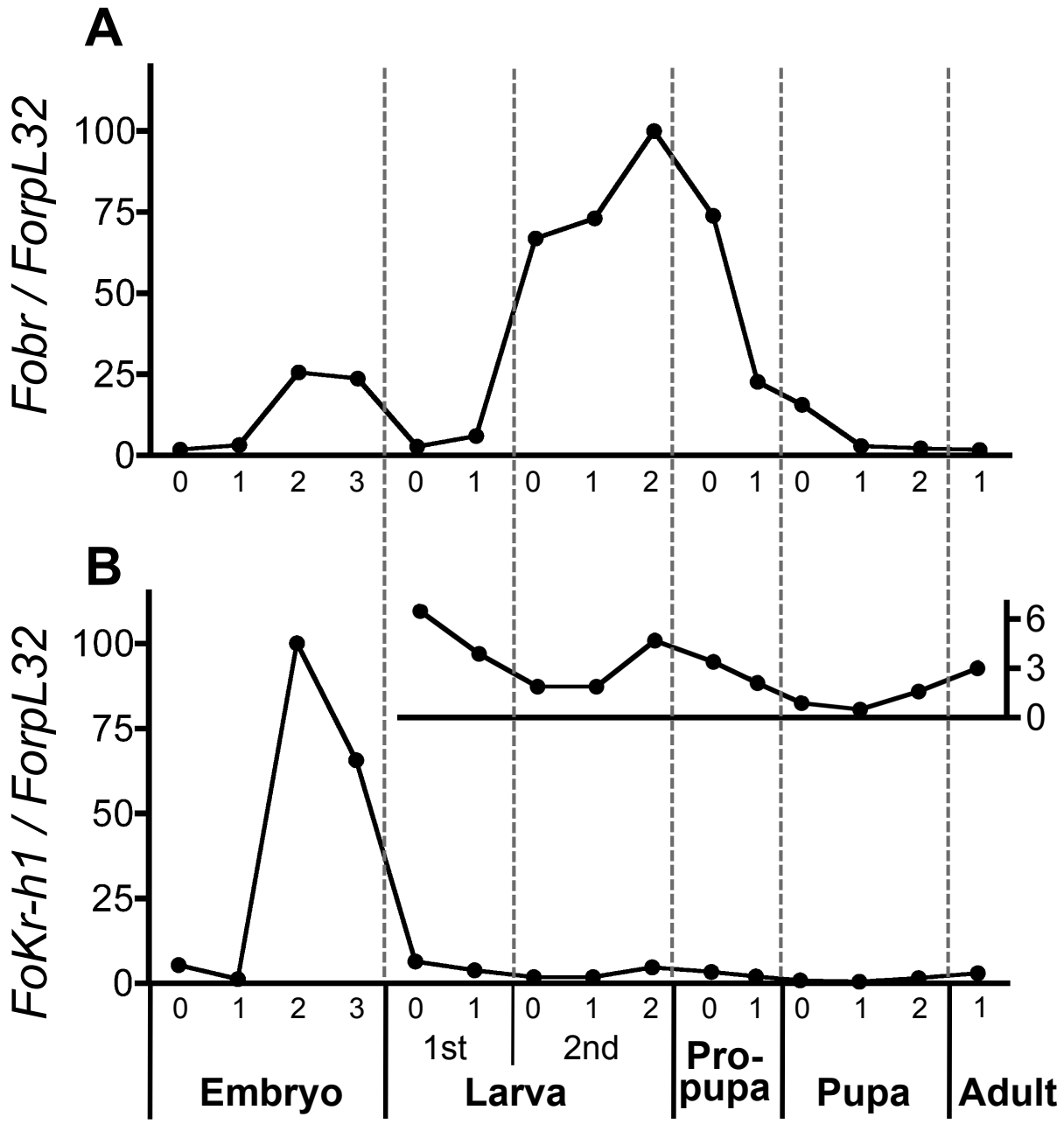


Figure3C

Fig. 3.

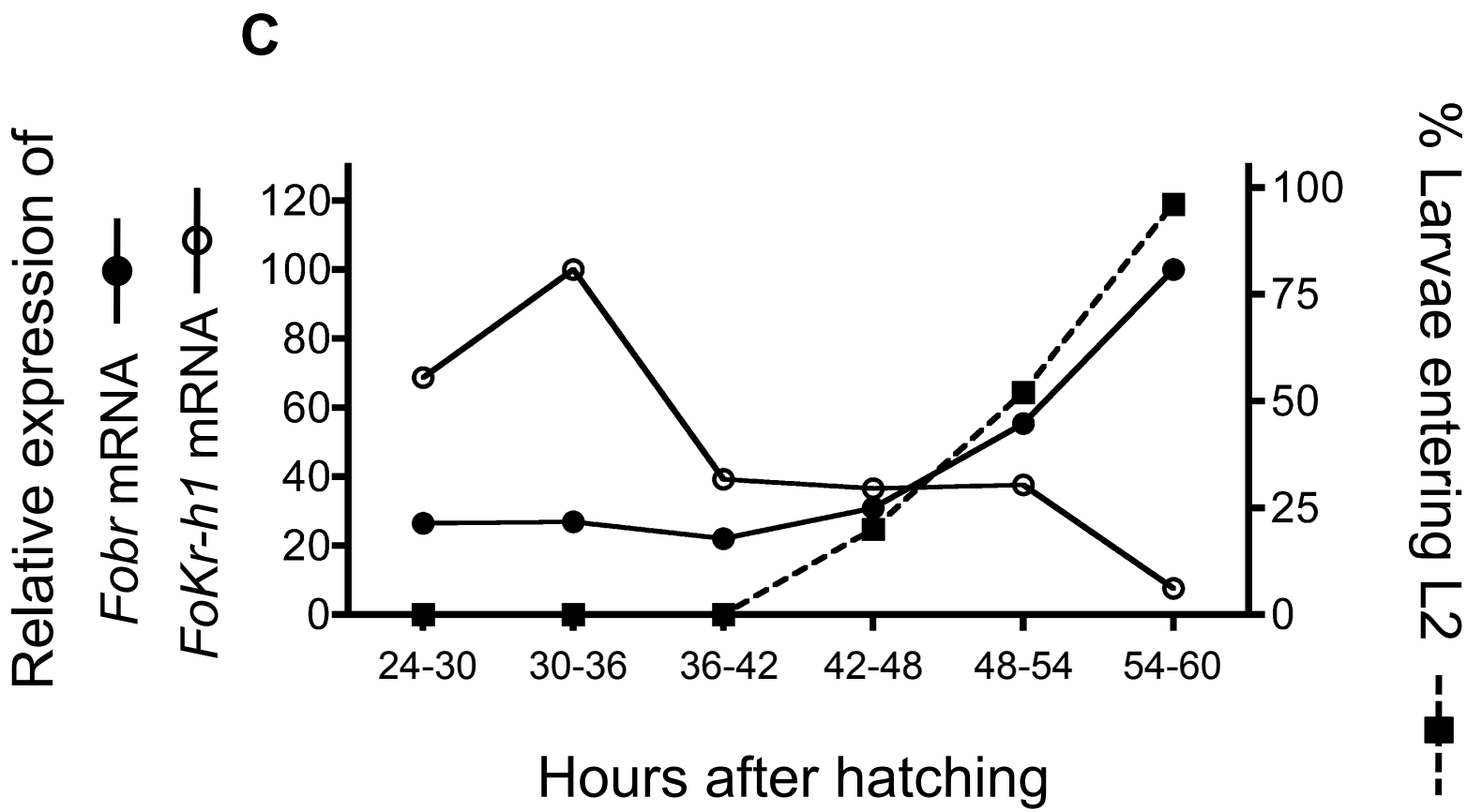


Figure4

Fig. 4.

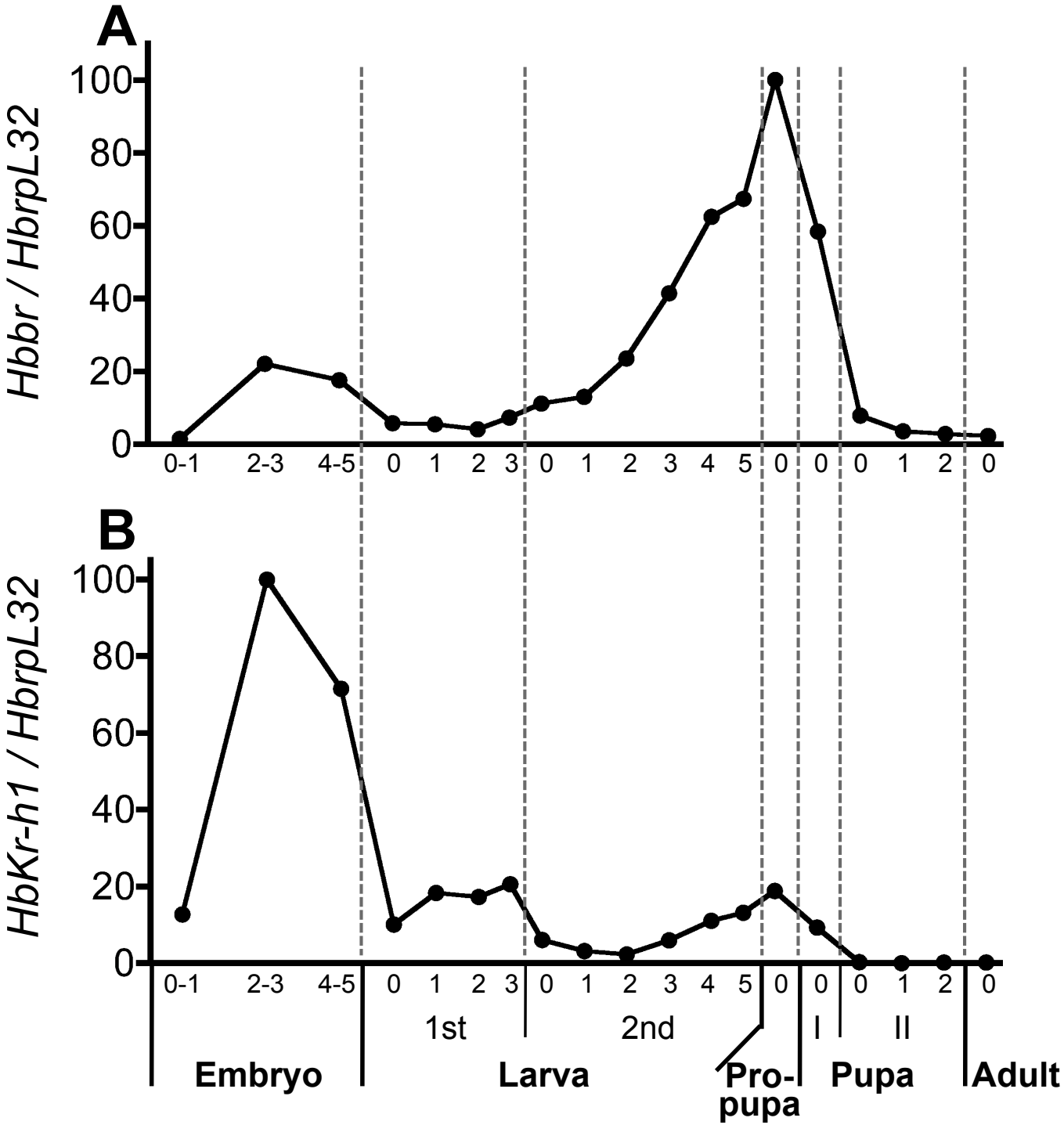


Figure5

Fig. 5.

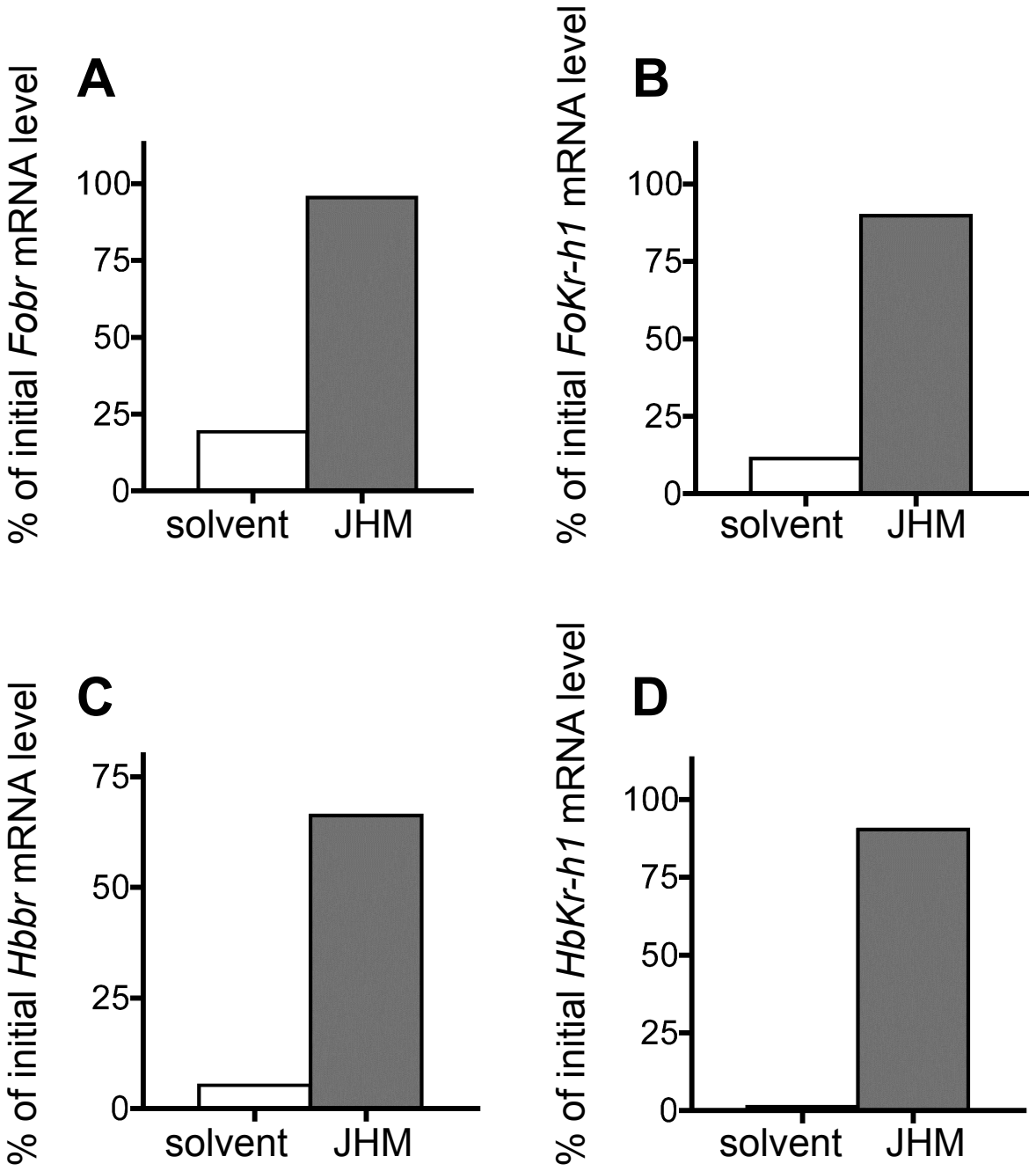
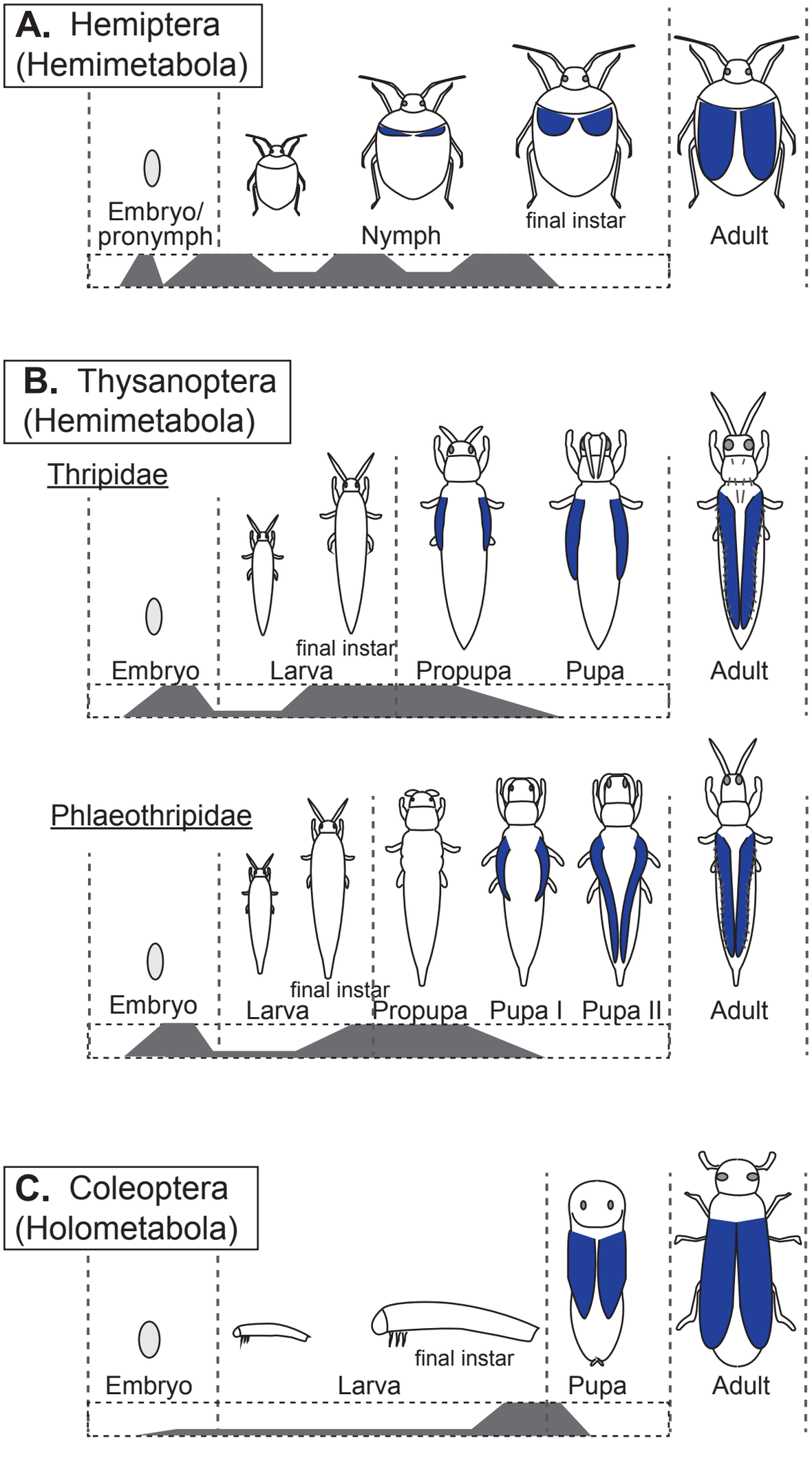


Fig. 6.


A. Hemiptera
(Hemimetabola)
Embryo/
pronymph
Nymph
final instar
Adult
B. Thysanoptera
(Hemimetabola)
Thripidae
Embryo
Larva
final instar
Propupa
Pupa
Adult
Phlaeothripidae
Embryo
Larva
final instar
Propupa
Pupa I
Pupa II
Adult
C. Coleoptera
(Holometabola)
Embryo
Larva
final instar
Pupa
Adult