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The MEKRE93 (Methoprene tolerant-Krüppel homolog 1-E93) pathway in the regulation of insect metamorphosis, and the homology of the pupal stage

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ABSTRACT

Recent studies on transcription factor E93 revealed that it triggers adult morphogenesis in *Blattella germanica*, *Tribolium castaneum* and *Drosophila melanogaster*. Moreover, we show here that Krüppel homolog 1 (Kr-h1), a transducer of the antimetamorphic action of juvenile hormone (JH), represses E93 expression. Kr-h1 is upstream of E93, and upstream of Kr-h1 is Methoprene-tolerant (Met), the latter being the JH receptor in hemimetabolan and holometabolan species. As such, the Met – Kr-h1 – E93 pathway (hereinafter named "MEKRE93 pathway") appears to be central to the status quo action of JH, which switch adult morphogenesis off and on in species ranging from cockroaches to flies. The decrease in Kr-h1 mRNA and the rise of E93 expression that triggers adult morphogenesis occur at the beginning of the last instar nymph or in the prepupae of hemimetabolan and holometabolan species, respectively. This suggests that the hemimetabolan last nymph (considering the entire stage, from the apolysis to the last instar until the next apolysis that gives rise to the adult) is ontogenetically homologous to the holometabolan pupa (also considered between two apolyses, thus comprising the prepupal stage).

1. Hormones and metamorphosis

Two principal hormones are involved in insect metamorphosis. One of these is molting hormone (MH), which promotes the successive molts that allow growth and postembryonic development, and the other is juvenile hormone (JH), which represses metamorphosis (Nijhout, 1994). According to Wigglesworth (1965), Hachlow was the first to discover MH to be a hormone originating in the thorax, by means of ligature experiments carried out on a number of papilionid species in 1931. At the time, however, the conclusion to be drawn from this was confused since it was unclear whether Hachlow's MH was the same as, or different from the brain-derived molting promoting factor discovered in the gypsy moth by Stephan Kopeč in 1917. Thereafter, Wigglesworth observations in the blood-sucking bug *Rhodnius prolixus* of what we now know to be ecdysone allowed to distinguish the thoracic molting factor from the brain molting factor, and show that both existed in *R. prolixus* (Wigglesworth, 1934). This contribution, however, mistakenly proposed that the thoracic molting hormone probably originated in the corpus allatum (CA). Of note, Wigglesworth's research was more or less coincident with the work published by Fraenkel in 1934 and 1935, which showed that neck ligature of mature larvae of *Calliphora* blowflies gave as result that only the head part formed a puparium, whereas the remaining body region kept larval features. The famous experiments of parabiosis reported by Wigglesworth (1940) consolidated the idea of a two-stage hormonal control of molting, and the same year Fukuda established that MH is secreted by the prothoracic glands, not by the CA. The relative contributions of the brain and thorax hormones to the initiation of molting was resolved by Piepho, who in 1942 suggested that the brain secreted a trophic hormone that stimulated the production of the actual stimulus to molt. Concerning JH, in his 1934 paper Wigglesworth identified the source of a hormone derived from the head which inhibited metamorphosis, and the parabiosis experiments of the 1940 paper confirmed the occurrence of this hormone circulating in the haemolymph. Practically in parallel, the experiments on silkworms reported by Bounhiol in 1938, clearly showed that that the source of the inhibitory hormone was the CA (Karlson, 1996; Wigglesworth, 1985).

The chemical structure of MH was elucidated in 1965 by Karlson and co-workers, who used extracts of pupae from the silkworm *Bombyx mori*. MH turned out to be a highly hydroxylated steroid

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that was named ecdysone, although soon after the structural elucidation of ecdysone it was realized that an ecdysone derivative further hydroxylated at position 20, or 20-hydroxyecdysone (20E), was the biologically active hormone in most cases (Karlson, 1996). Chemical analysis of the first JH was achieved by Röller and co-workers in 1967 using extracts form the Cecropia moth, *Hyalophora cecropia*. This analysis revealed that it had a terpenoid structure, which was quite extraordinary for a hormonal compound (Röller and Dahm, 1968). Both hormones were soon synthetically available, thus facilitating the study of their biological properties and leading to rapid progress in the field of insect endocrinology, especially concerning basic physiological aspects (Karlson, 1996; Wigglesworth, 1985).

The next step was to investigate the molecular action of the hormones, beginning with 20E, the steroid nature of which allowed inspiring references from the vertebrate steroid world to be found. In this sense, the paper of Clever and Karlson (1960), which established that ecdysone action was "to alter the activity of specific genes", was seminal and subsequent results published by Clever during the 1960s confirmed this kind of action. These results were essential to inspire Ashburner to produce his now famous model in which binding of 20E to its receptor induces the expression of early genes whose products repress the expression of their own genes and induce that of late genes, thus leading to the final genes and the corresponding effector proteins (Ashburner et al., 1974).

As demonstrated in *Drosophila melanogaster*, the 20E receptor turned out to be a heterodimer of two nuclear receptors: the ecdysone receptor, or EcR, described as such by Hogness and his group in 1991, and ultraspiracle, or USP, whose involvement in 20E reception was reported by Evans and co-workers in 1992 and 1993. Step by step, and using *D. melanogaster* as model, a great deal of information on the 20E signalling pathway was soon obtained, including the identification of most transcription factors operating in it, many of which belong to the nuclear receptor superfamily, and whose epistatic relationships generally fitted well with the Ashburner model (King-Jones and Thummel, 2005; Nakagawa and Henrich, 2009).

2. The case of broad complex

Broad complex (BR-C) is a family of transcription factors with a bric-à-brac/tramtrack/broad (BTB) motif in the N-terminal region and two C2/H2 zinc fingers in the C-terminal region that can bind to DNA (Bayer et al., 1996; DiBello et al., 1991). BR-C expression results in a number of different isoforms that differ in the zinc fingers. Intriguingly, a phylogenetically basal species such as the German cockroach, *Blattella germanica*, expresses six isoforms (Piulachs et al., 2010), whereas *D. melanogaster*, which is a highly derived holometabolan species, expresses only four (DiBello et al., 1991). BR-C is a well-known early response gene in the 20E signalling pathway whose expression is also influenced by JH, although this influence is very different in hemimetabolan and holometabolan species. Thus, in hemimetabolan species, JH stimulates BR-C transcription during early nymphal stages and mRNA levels only decay in the last nymphal instar, when circulating JH vanishes (Huang et al., 2013; Konopova et al., 2011) (Fig. 1). In contrast, in holometabolan species JH inhibits BR-C expression during larval stages, thus meaning that the corresponding mRNAs are upregulated in the last larval instar, when a small peak of 20E produced in the absence of JH (the commitment peak) determines the formation of the pupa. Interestingly, levels of BR-C expression are low in the two first larval instars of the JH deficient mutant *dimolting* (*mod*) of *B. mori* (Daimon et al., 2012; Smykal et al., 2014), which suggest that BR-C expression in these two first instars is JH-independent. From the commitment peak on, JH no longer

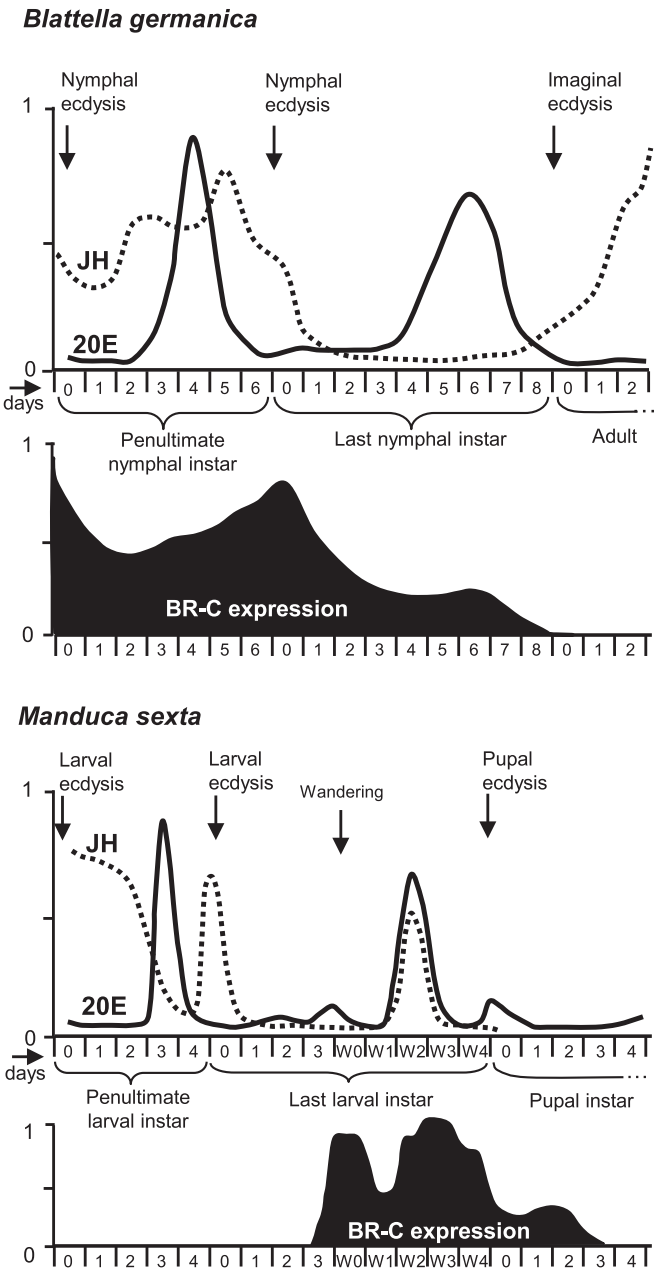

Fig. 1. Juvenile hormone (JH) and 20-hydroxyecdysone (20E) concentration profiles in the haemolymph, and expression of Broad Complex (BR-C) transcription factors in the last developmental instars in a hemimetabolan (the cockroach *Blattella germanica*) and a holometabolan (the moth *Manduca sexta*) species. Units in the ordinates only reflect the relative levels at each moment, taking the maximal values measured for each species as equal to 1.Original values of hormone titers of *B. germanica* can be found in Romaña et al. (1995) (20E) and Treiblmayr et al. (2006) (JH), and those of BR-C in Huang et al. (2013). Original values of hormone titers of *M. sexta* can be found in Riddiford et al. (2003).

inhibits but now stimulates BR-C transcription, thus BR-C expression increases with the increase of JH production in the prepupal stage (Zhou and Riddiford, 2002) (Fig. 1).

Functional studies of BR-C in hemimetabolan species, such as the cockroach *B. germanica* and the bugs *Oncopeltus fasciatus* and *Pyrrhocoris apterus*, have shown that the different isoforms of BR-C contribute to the regulation of the progressive development of wing primordia during juvenile stages (Erezyilmaz et al., 2006; Huang et al., 2013; Konopova et al., 2011). Detailed studies

carried out in *B. germanica* have shown that BR-C transcription factors maintain high levels of cell proliferation in wing primordia during juvenile stages, and that RNAi depletion of BR-C mRNAs lead to adults with small wings and tegmina, and with minor defects in forewing venation (Huang et al., 2013). Further studies have suggested that BR-C action on wing venation might be mediated by the microRNAs let-7, miR-100 and miR-125 (Rubio and Belles, 2013). In contrast, functional studies of BR-C performed in holometabolan species, such as the fly *D. melanogaster*, the moths *Manduca sexta* and *B. mori* and the beetle *Tribolium castaneum*, have shown that BR-C proteins are required to trigger the pupal stage (Konopova and Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008; Uhlirova et al., 2003; Zhou and Riddiford, 2002). In *T. castaneum*, however, it became clear that specimens depleted for BR-C in the last larval instar molted to individuals with mixed features of larva and pupa and, intriguingly, of adult, especially in the antennae, mandibles and the cuticle in general (Konopova and Jindra, 2008; Parthasarathy et al., 2008). This indicated that BR-C factors trigger the formation of pupae while repressing adult morphogenesis, at least in certain tissues.

3. The enigma of the JH signalling pathway begins to be unveiled

In contrast to the 20E signalling pathway, the JH signalling pathway remained essentially a complete enigma until very recently, when a number of findings have determined that the receptor for JH is a protein called Methoprene tolerant, or Met (Jindra et al., 2013). Met was discovered by Wilson in 1986 as a factor that determines resistance towards the antimetamorphic effects of JH and JH analogues (like methoprene) in some *D. melanogaster* strains (Ashok et al., 1998; Wilson and Fabian, 1986). In 2005, it was shown that, when translated *in vitro*, *D. melanogaster* Met protein could bind JH with very high affinity (Kd of 5.3 nM) (Miura et al., 2005), thereby suggesting that Met might play a role in JH reception. An elegant functional proof that Met was involved in transducing the antimetamorphic JH signal was obtained two years later by depleting Met expression in the early larval stages of *T. castaneum* with RNAi and observing that they precociously metamorphosed (Konopova and Jindra, 2007). More recently, RNAi studies have shown the same role in hemimetabolan species such as *P. apterus* (Konopova et al., 2011) and *B. germanica* (Lozano and Belles, in press).

Structurally, Met is a protein belonging to the bHLH (basic-helix-loop-helix)/PAS (Per-Arnt-Sim) family. The bHLH domain is located at the N-terminal end, after a short stretch of basic amino acids, followed by two PAS motifs (A and B). To become activated, the bHLH/PAS proteins bind to another protein often from the same family, forming a heterodimer which is then able to bind DNA. Both the bHLH domain and the two PAS motifs participate in this dimerization, whereas the C-terminal region possesses transactivating functions. The PAS motifs are involved in protein–protein interactions, allow subcellular translocation, and recognize ligands (Jindra et al., 2013). Using Met of *T. castaneum*, Charles et al. (2011) have confirmed that JH III binding affinity is high (Kd of 2.9 nM in this case), that the PAS-B motif is necessary and sufficient to bind JH, and that when JH binds to a Met moiety in a Met–Met homodimer, the homodimer dissociates and the Met + JH complex binds to another bHLH/PAS protein called Taiman, thus suggesting that binding to JH triggers a conformational change that makes Met accessible to other proteins (Charles et al., 2011). *D. melanogaster* possesses two Met paralogues (Met and Germ-cell expressed, GCE) with partially redundant roles, which precluded clear results regarding their function in metamorphosis, as Met-null mutants were completely viable. Sequence comparison studies have shown that GCE is more similar to Met of other species than *D. melanogaster* Met (Baumann et al., 2010).

Downstream of Met, the JH signal is transduced by Krüppel homolog 1 (Kr-h1), a transcription factor with a DNA binding domain of eight zinc fingers. The expression pattern of Kr-h1 is peculiar because its mRNA is present in juvenile instars, but decrease at the beginning of the pre-adult stage, as observed in prepupae of holometabolan species, such as *D. melanogaster* (Minakuchi et al., 2008) and *T. castaneum* (Minakuchi et al., 2009), and in the last nymphal instar of hemimetabolan species, such as *P. apterus* (Konopova et al., 2011) and *B. germanica* (Lozano and Belles, 2011) (Fig. 2). Kr-h1 expression also falls in pre-adult stages in thrips (Thysanoptera) (Minakuchi et al., 2011) (Fig. 2), which show a particular type of hemimetaboly known as neometaboly (Sehnal et al., 1996) and whose life cycle includes two or three pre-adult stages that are reminiscent of the holometabolan pupae.

The decrease in Kr-h1 expression at the beginning of the pre-adult stage coincides with the gradual decrease of JH concentration in the haemolymph, as observed, for example, in *B. germanica*, where JH titers and Kr-h1 mRNA levels have been precisely measured (Lozano and Belles, 2011; Treiblmayr et al., 2006) (Fig. 2). Moreover, treatment with JH at this pre-adult stage re-induced Kr-h1 expression and inhibited adult morphogenesis in *P. apterus* and *B. germanica* (Konopova et al., 2011; Lozano and Belles, 2011), and dsRNA targeting Kr-h1 applied prior to JH analogue treatment in the last nymphal instar of *P. apterus* rescued normal adult morphogenesis, averting the supernumerary nymphal instar that is obtained when applying the JH analogue alone (Konopova et al., 2011). These observations suggest that the decrease in Kr-h1 expression at the beginning of the pre-adult stage is important for metamorphosis and that it is primarily triggered by a decrease of circulating JH and indicate that JH induces Kr-h1 expression. The direct induction of Kr-h1 expression by JH and Met has been well documented in several systems, but a recent work of Kayukawa et al. (2014) has provided an elegant genetic gain-of-function evidence for the repressive role of Kr-h1 on metamorphosis. When misexpressed constitutively in transgenic *B. mori*, Kr-h1 was able to disrupt pupation, although it was not sufficient to completely maintain the larval character of the upcoming molt (Kayukawa et al., 2014). Moreover, studies carried out in *B. mori* by the same group have shown that the promoter region of the Kr-h1 gene contains a motif that can bind bHLH/PAS proteins, thereby suggesting that the Met-JH/Taiman complex binds to this promoter region to induce Kr-h1 expression (Kayukawa et al., 2012).

From a functional point of view, the properties of Kr-h1 as a transducer of the antimetamorphic action of JH has been shown in both hemimetabolan species, such as *B. germanica* (Lozano and Belles, 2011), *P. apterus* and *R. prolixus* (Konopova et al., 2011), as well as in holometabolan species, such as *D. melanogaster* (Minakuchi et al., 2008), *T. castaneum* (Minakuchi et al., 2009) and *B. mori* (Kayukawa et al., 2014; Smykal et al., 2014). All these data dramatically demonstrate that Kr-h1 is a master repressor of adult morphogenesis, but it is remarkable that Kr-h1 RNAi do not induce adult morphogenesis in early nymphal or larval instars, and that reduction of Kr-h1 mRNA levels did not lead to pupation in early instars of *mod B. mori* mutants. This suggests that insects acquire competence to metamorphose only in late nymphal or larval instars (Smykal et al., 2014).

4. Downstream Kr-h1: E93 emerges

While Kr-h1 appears to be a sort of master repressor of metamorphosis conserved in hemimetabolan and holometabolan species, what is the immediate target of Kr-h1? A plausible hypothesis would be that Kr-h1 was repressing a factor that promotes adult

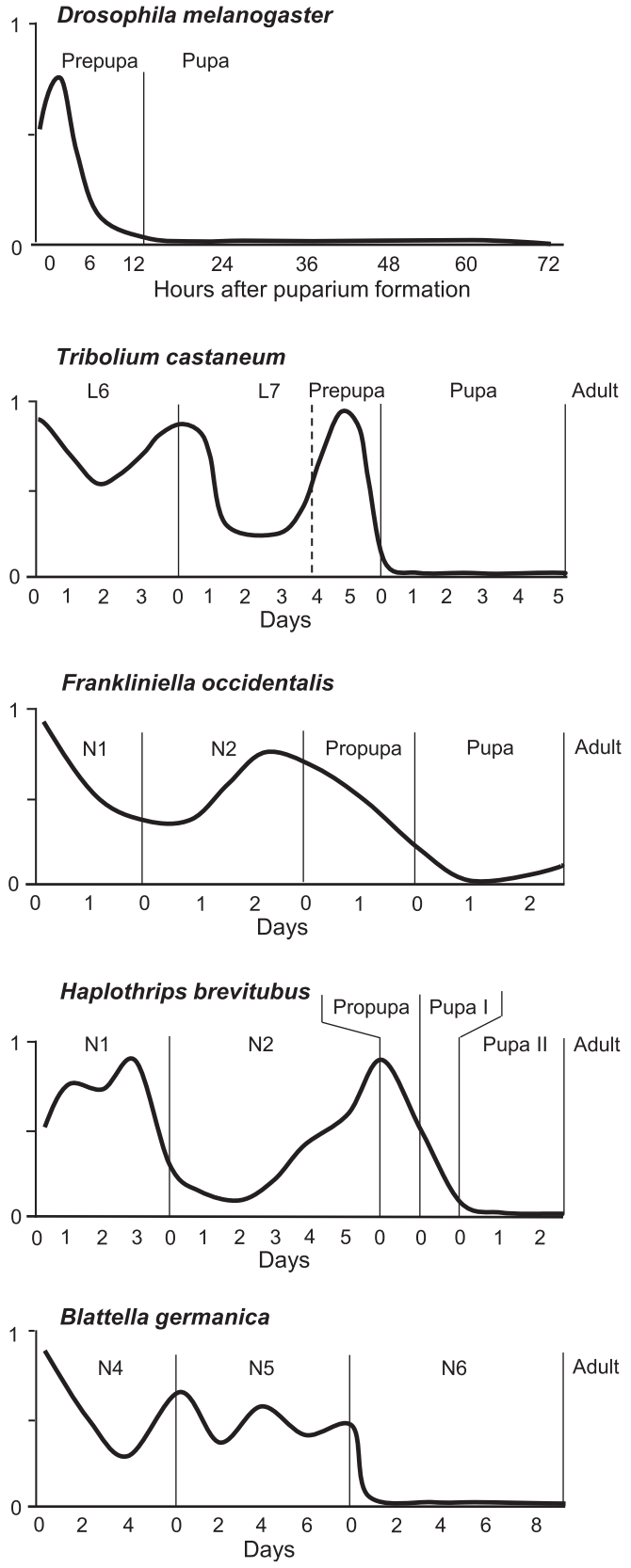

Fig. 2. Expression profiles of Kr-h1 during the last juvenile stages in *Drosophila melanogaster* (Minakuchi et al., 2008), *Tribolium castaneum* (Minakuchi et al., 2009), *Frankliniella occidentalis* and *Haplothrips brevitubus* (Minakuchi et al., 2011), and *Blattella germanica* (Lozano and Belles, 2011). Units in the ordinates only reflect the relative mRNA levels at each moment, taking the maximal values measured for each species as equal to 1.Original values can be found in the respective references. Larval and nymphal instars are abbreviated as L and N, respectively.

morphogenesis, and a recent paper by Ureña et al. (2014) strongly suggests that the ecdysteroid-dependent transcription factor Eip93F, more familiarly known as E93, fits this role.

E93 is a helix-turn-helix transcription factor containing a Pip-squeak (Psq) motif, which was first reported to be involved in the regulation of programmed cell death in *D. melanogaster* prepupae (Baehrecke and Thummel, 1995; Lee et al., 2000). Later, an E93 homolog called Mblk-1 was discovered in the large-type Kenyon cells of the mushroom bodies of *Apis mellifera* brain, which suggested its involvement in the advanced behaviours of honeybees (Takeuchi et al., 2001). A neat hint that E93 might be related to adult morphogenesis was afforded by Jafari et al. (2012) and Mou et al. (2012), who showed that it appeared to be a more general regulator of gene expression in *D. melanogaster* metamorphosis. Ureña et al. (2014) identified E93 when looking for genes controlling apoptosis in prothoracic glands and morphogenesis in wings of the hemimetabolan species *B. germanica* during metamorphosis, and they showed that its depletion by RNAi in preimaginal stages prevented formation of the adult in both *B. germanica* and in the holometabolan species *T. castaneum* and *D. melanogaster*. In all three species, E93 is specifically expressed in the preimaginal stage, that is, in the last nymphal instar of *B. germanica* and in the prepupae and pupae of *T. castaneum* and *D. melanogaster* (Ureña et al., 2014). In *B. germanica*, E93 RNAi treatment carried out on penultimate (N5) or last (N6) instar female nymphs effectively depleted E93 mRNA levels in N6 and prevented metamorphosis to the adult stage. Instead, the specimens molted to a perfect supernumerary (N7) nymph. Of note, specimens that were RNAi-treated in N6 (which had the normally low levels of Kr-h1 at the beginning of this stage), molted to a supernumerary N7, which suggests that high levels of Kr-h1 are not a sufficient condition to promote adult morphogenesis. Indeed, this is coherent with the results obtained in *B. mori* through experiments of misexpression of Kr-h1 in the last larval instar (Kayukawa et al., 2014). Ureña et al. (2014) also depleted E93 expression in pupae of *T. castaneum* and *D. melanogaster*, which prevented adult morphogenesis, and the specimens molted to supernumerary pupae, which were not able to completely ecdyse (*T. castaneum*), or died as pupae without molting (*D. melanogaster*).

Ureña et al. (2014) carried out additional experiments on *B. germanica* with the JH analogue methoprene, administering the compound on day 3 of the penultimate instar (N5D3) at doses of 500, 100 and 10 nM, and measuring the expression of E93 and Kr-h1 in N6D1. Results showed that E93 levels were on average 35% lower in those insects treated with the highest dose of methoprene (40% lower at 100 nM, and 16% lower at 10 nM). Conversely, there was a clear dose-dependent action of methoprene on Kr-h1 expression, with Kr-h1 mRNA levels very high (similar to those in N5) in the specimens treated with 500 nM, medium in the 100 nM-treated specimens, and very low (as in control N6D1) in those treated with 10 nM. Interestingly, only the dose of 500 nM of methoprene (associated to high expression of Kr-h1 and medium expression of E93) produced supernumerary nymphs (Ureña et al., 2014). This suggests that E93 expression must surpass a given threshold and reach optimal levels in order to effectively promote adult differentiation.

An interesting feature of Kr-h1 expression in *B. germanica* is that it abruptly decreases in freshly emerged N6, as reported by Lozano and Belles (2011) (Fig. 2). However, Ureña et al. (2014) showed that Kr-h1 expression did not decrease properly in specimens where E93 had been depleted, which suggests that E93 contributes to downregulate Kr-h1 in this stage. In *B. germanica*, haemolymph JH decreases from ca. 5 ng/ml to undetectable levels within the first 24 h of the last nymphal instar (Treiblmayr et al., 2006), while within the same 24 h-period Kr-h1 mRNA levels dramatically

decrease (Lozano and Belles, 2011) and E93 expression increase in metamorphic tissues (Ureña et al., 2014). Given that the first signs of up-regulation of E93 expression appear to occur before the decrease of JH and Kr-h1, Ureña et al. (2014) propose that they are triggered by JH-independent factors, like the attainment of a critical size, a mechanism that might operate specifically at the beginning of the last nymphal instar, when the genetic program for adult morphogenesis is triggered.

Taken as a whole, these data suggest to us that a decrease of JH levels would result in a primary decrease of Kr-h1 expression until reaching a given threshold that would lead, by de-repression, to full expression of E93. The onset of E93 expression may had been triggered by the attainment of a critical size and mediated by ecdysteroid signalling, as E93 is an ecdysteroid-dependent gene. Subsequently, full expression of E93 would contribute to down-regulate Kr-h1 expression. Indeed, a repressor action of Kr-h1 upon E93 operates in the penultimate nymphal instar, when depletion of Kr-h1 by RNAi triggers metamorphosis precociously (Lozano and Belles, 2011) through up-regulation of E93 (see next section). In this sense, the competence of juvenile stages to metamorphose (Smykal et al., 2014), may be mediated by the competence of these stages to express E93.

Finally, Ureña et al. (2014) showed that E93 depletion in *T. castaneum* and *D. melanogaster* prevented the BR-C down-regulation that normally occurs during the pupal stage and is crucial for adult morphogenesis. Intriguingly, E93 depletion also prevented the BR-C downregulation during the last nymphal instar of *B. germanica* (Ureña et al., 2014), although BR-C does not appear to repress adult morphogenesis in hemimetabolan species.

5. Kr-h1 represses E93 expression

In an attempt to set up a sort of minimal model of metamorphosis, in our laboratory we decided to focus on the morphogenesis of tergal glands on male cockroaches, which are complex structures that secrete pheromones that serve to attract a female and to facilitate mating (Roth, 1969). In *B. germanica*, the tergal glands form de novo in tergites 7 and 8 in the transition from the last nymphal stage to adult, and their morphology has been studied by Sreng and Quennedey (1976). In order to find factors regulated by Kr-h1, we compared transcriptomes from male tergites 7 and 8 (MT7-8), from *B. germanica* N6D1 (transcriptome T-N6D1), at which point Kr-h1 expression has practically vanished (Lozano and Belles, 2011), and MT7-8 from male N6D1 treated with 20 μg of JH III, 24 h previously (transcriptome T-N6D1+JH) (Ylla and Belles, unpublished). As JH treatment upregulates Kr-h1 expression (Lozano and Belles, 2011), we assumed that transcripts depleted in T-N6D1+JH compared with T-N6D1 might correspond to factors repressed by Kr-h1, and E93 was one of the transcripts that appeared significantly depleted.

Our unpublished new data (Fig. 3) shows that E93 expression in MT7-8 increases at the beginning of male N6 while Kr-h1 mRNA levels decreases (Fig. 3A). Moreover, our experiments in which E93 was depleted by RNAi in male N6 (Fig. 3B) resulted in inhibition of metamorphosis and triggering the formation of supernumerary N7 nymphs (Fig. 3C), whereas the decrease of Kr-h1 that normally occurs at the beginning of this stage was prevented (Fig. 3D), just as observed in female nymphs by Ureña et al. (2014). In our experiments, the supernumerary (N7) male nymphs metamorphosed to adults at the next molt, thus suggesting that E93 mRNA levels started to recover, as was the case (Fig. 3E), whereas those of Kr-h1 remained low, as expected (Fig. 3E). Comparison of the Kr-h1 and E93 expression patterns (Fig. 3A) suggested to us that Kr-h1 may repress E93 expression. To test this hypothesis, we carried out the new experiment of depleting Kr-h1 mRNA levels by RNAi in N5D0, following the methodology described by Lozano and Belles (2011), and measuring E93 (and Kr-h1) mRNA levels in N5D4 and N5D6. Results indicated that decreasing Kr-h1 mRNA levels correlate with increasing E93 mRNA levels (Fig. 3F). This new experiment affords the key information that Kr-h1 represses E93 expression.

Interestingly, the decrease in Kr-h1 mRNA levels runs in parallel to the increase of E93 expression in *B. germanica*, *T. castaneum* and *D. melanogaster* (Ureña et al., 2014; present results) (Fig. 4A), and the signalling pathways that trigger formation of the last nymphal instar and then determine the fate of the adult in a hemimetabolan insect like *B. germanica* (Fig. 4B) appear equivalent to those operating in a holometabolan species like *T. castaneum* (Fig. 4C). A repressor role of Kr-h1 upon E93 in *T. castaneum* and *D. melanogaster* can be predicted on the basis of the results herein described for *B. germanica*, and considering the properties of Kr-h1 as a transducer of the antimetamorphic action of JH reported in *T. castaneum* (Minakuchi et al., 2009) and *D. melanogaster* (Minakuchi et al., 2008), and the essential role of E93 for adult morphogenesis in these holometabolan species (Ureña et al., 2014). The key position of E93 in the signalling pathway can also help us to better understand the role of BR-C. Indeed, the intriguing formation of adult features in the specimens emerging from BR-C-depleted last instar larvae of *T. castaneum* (Konopova and Jindra, 2008; Parthasarathy et al., 2008) could be the result of a repressor action of BR-C on E93 expression in the transition from prepupae to the pupae (Fig. 4C). This inference would be compatible with the decrease in BR-C mRNA levels (Konopova and Jindra, 2008; Parthasarathy et al., 2008) that occurs in parallel to the increase in E93 mRNA levels (Ureña et al., 2014) observed at the beginning of the pupal stage of *T. castaneum*.

6. The MEKRE93 pathway and the homology of the pupal stage

The discovery of E93 as a trigger of adult metamorphosis in *B. germanica*, *T. castaneum* and *D. melanogaster* by Ureña et al. (2014) indicates that this function of E93 is conserved at least from cockroaches to flies. Moreover, the unpublished data presented herein indicate that Kr-h1 represses E93 expression. Kr-h1 is upstream of E93, and the function of Kr-h1 as transducer of the antimetamorphic action of JH has also been demonstrated in hemimetabolan and holometabolan species (Kayukawa et al., 2014; Konopova et al., 2011; Lozano and Belles, 2011; Minakuchi et al., 2009, 2008). Finally, Met, the function of which as a JH receptor has also been shown to be conserved in hemimetabolan and holometabolan insects, is upstream of Kr-h1 (Konopova and Jindra, 2007; Konopova et al., 2011). With this in mind, the basic pathway Met - Kr-h1 - E93 (herein named “MEKRE93 pathway” for simplification) appears to be central to the status quo action of JH, transducing the hormonal signal that switches adult morphogenesis off and on. Importantly, this pathway is conserved across the Hexapoda, from cockroaches (basal hemimetabolan) to beetles (basal holometabolan) and flies (distal holometabolan), thus possibly predating the emergence of winged insects and the innovation of metamorphosis, more than 350 million years ago (Grimaldi and Engel, 2005). Additionally, the MEKRE93 pathway, together with classical morphological and physiological criteria, appears to provide an important clue for establishing homologies between insect life-cycle stages.

As stated above, the general expression patterns show that the decrease in Kr-h1 mRNA levels and the increase of E93 expression that determines adult morphogenesis both occur at the beginning of the last instar nymph or in the prepupae of hemimetabolan and holometabolan species, respectively (Fig. 4A), and the endocrine pathway that triggers the formation of the last nymphal stage of the

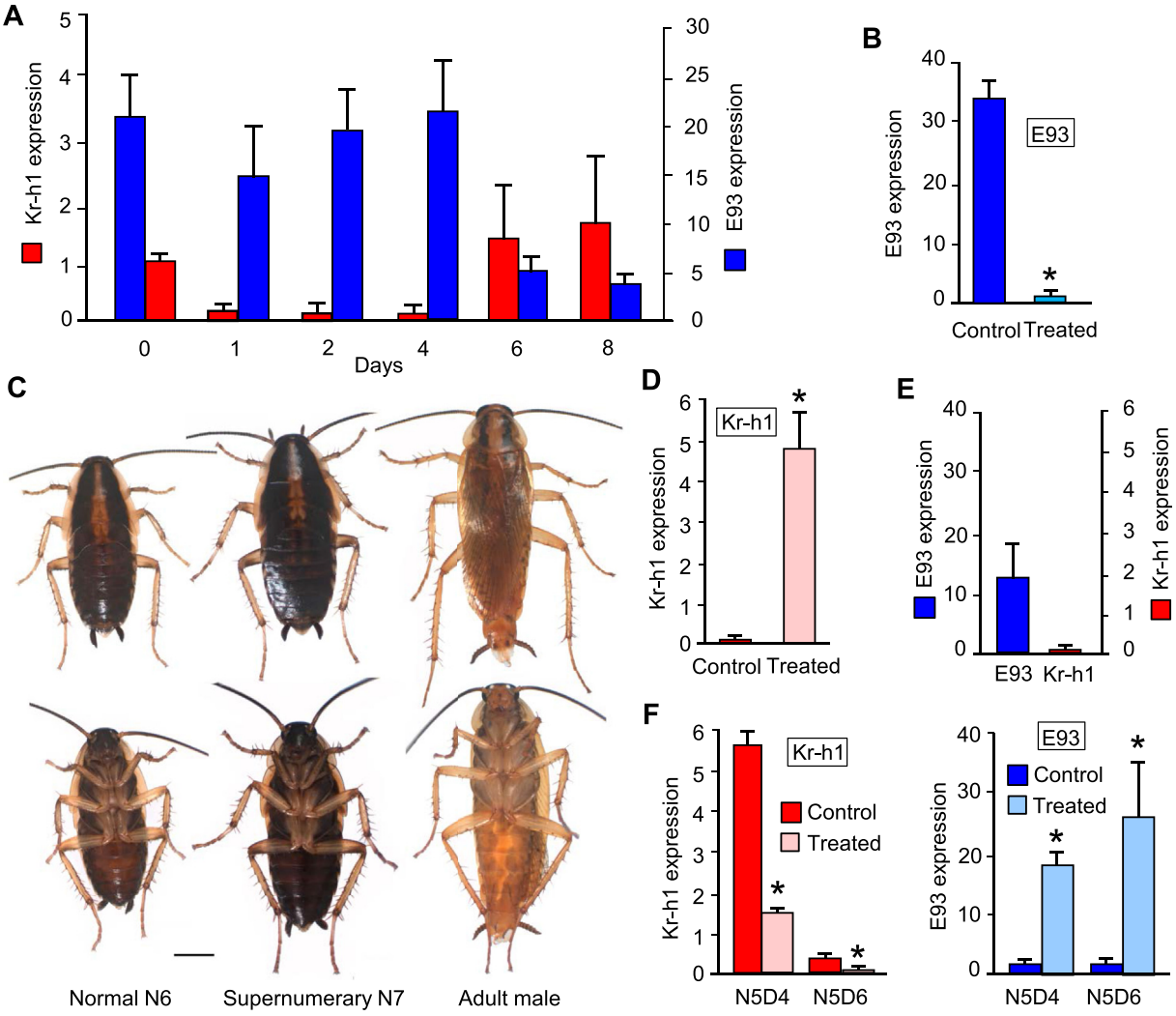

Fig. 3. Kr-h1 and E93 relationships in *Blattella germanica*. A. Expression of Kr-h1 and E93 in tergites 7 and 8 (MT7-8) of males during the sixth (last) nymphal instar (N6). B: RNAi treatment on day 0 of fifth nymphal instar (N5D0) males targeting E93; transcript decrease observed in N6D4 in MT7-8. C: Dorsal and ventral view of a supernumerary (N7) nymph obtained after the E93 RNAi treatment; a normal N6 and adult male are shown for comparison; scale bar: 2 mm. D: mRNA levels of Kr-h1 in E93 knockdowns; RNAi treatment targeting E93 was performed in N5D0, and Kr-h1 mRNA levels were measured in N6D4. E: mRNA levels of E93 and Kr-h1 in N7D4 in E93 knockdowns. F: mRNA levels of E93 in Kr-h1 knockdowns; RNAi treatment targeting Krh1 was performed in N5D0, then E93 (and Kr-h1) mRNA levels were measured in N5D4 and N5D6. In all cases, asterisks indicate significant differences of treated with respect to controls ($p < 0.05$), according to the REST software tool (Pfaffl et al., 2002). Insect rearing, RNA extraction and retrotranscription and quantification of mRNA levels with qRT-PCR, were as described by Lozano and Belles (2011), who detail the primers used to amplify Kr-h1 mRNA; those used to amplify E93 mRNA were: forward, 5- TCCAATGTTTGATCCTGCAA -3′ and reverse, 5′- GGATTTCTTTGCATCCCAAA-3′; results are given as copies of mRNA per 1000 copies of BgActin-5c mRNA, and expressed as mean ± standard error of the mean. General procedures for RNAi were as described by Lozano and Belles (2011), who detail the primers used to generate the dsRNA template to target Kr-h1. Those used in the case of E93 dsRNA were: forward: 5′-AAAGAGTTGTCGGGAGCAGA-3′ and reverse, 5′-GGAGTGGCTTCTAGCAGTGG-3′. As in previous works (Lozano and Belles, 2011), we used a sequence from *Autographa californica* nucleopolyhydrosis virus as control dsRNA (dsMock). A volume of 1 μl of DPEC water solution (containing 2–4 μg of dsRNA) was injected into the abdomen of specimens at chosen ages. The dsE93 treated males received two injections, one on N5D0 and the other on N6D0. The dsKrh1 treated samples received a single injection of on N5D0. Control specimens were equivalently treated with dsMock.

hemimetabolan species (Fig. 4B) looks very similar to that triggering the formation of the pupa of holometabolan species (Fig. 4C). Taken as a whole, these data suggest that the hemimetabolan last nymphal instar is ontogenetically homologous to the holometabolan pupa. More precisely, we should consider the entire last nymphal stage, from the moment of apolysis and secretion of the new cuticle that gives rise to the last nymphal instar (which occurs concomitantly with the peak of ecdysone at approximately 65–75% of the penultimate nymphal instar), until the next apolysis that will give rise to the adult. This last nymphal stage would be homologous to the pupal stage, also considered between two apolyses, which comprises the period of prepupae and the pupal stage proper (i.e. prior to the pharate adult stage). According to the Kr-h1 expression profiles (Fig. 2), the ensemble of the "propupa" and the two or three "pupal" stages of Thysanoptera (Neometabola) would be homologous to the last nymph of typical hemimetabolan species and to the holometabolan pupa. This implies the prediction that the expression of E93 in Thysanoptera would progressively increase during these stages concomitant to the decrease of Kr-h1 expression.

The homology of the pupa with the last nymphal instar is not a new idea, but it is in line with the proposals formalized by Hinton (1963) on the basis of morphological and life cycle data. This proposal later received support from physiological endocrinologists, who added physiological and hormonal evidence (Sehnal, 1985; Sehnal et al., 1996), and more recently by molecular

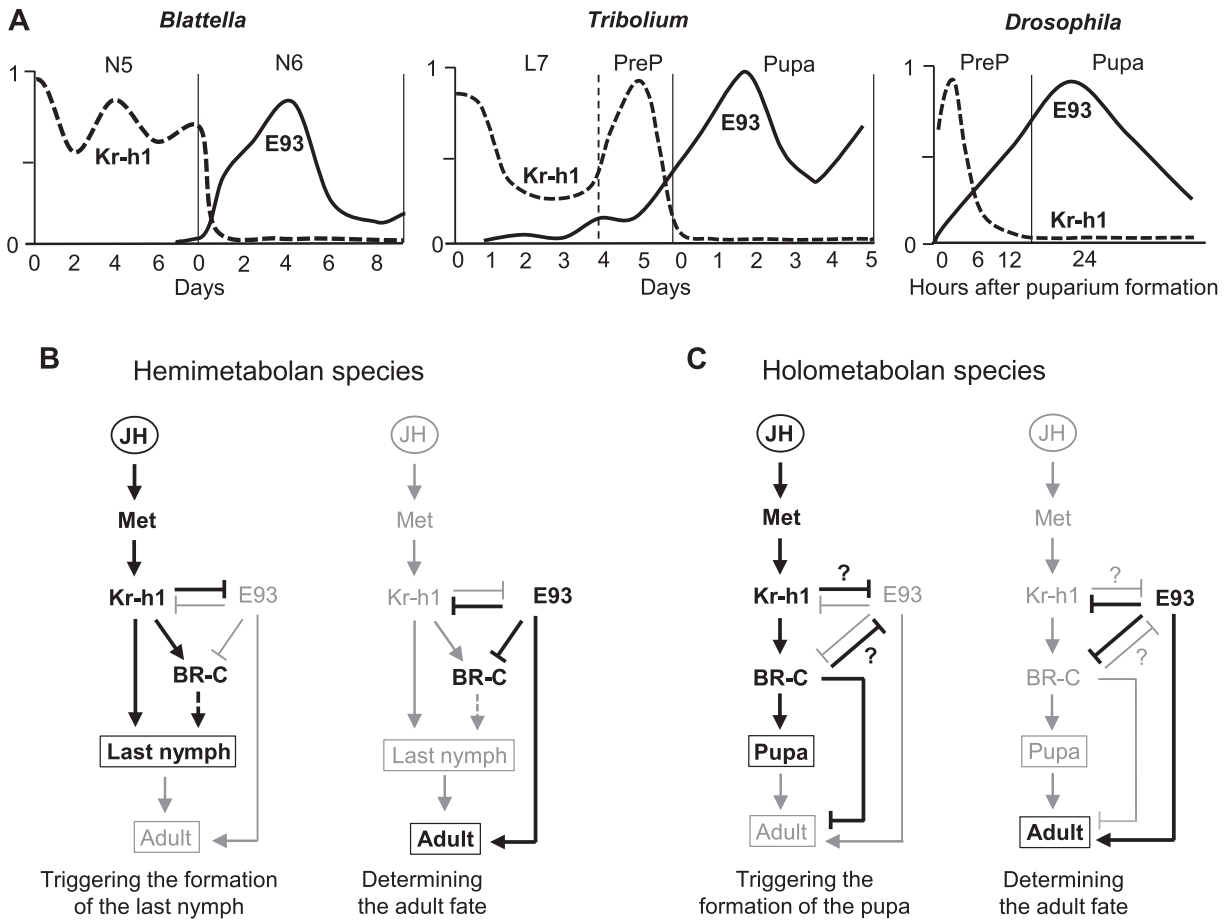

Fig. 4. A: Expression profiles of Kr-h1 and E93 during the pre-adult stages in *Blattella germanica*, *Tribolium castaneum* and *Drosophila melanogaster*. Units in the ordinates only reflect the relative mRNA levels at each moment, taking the maximal values measured for each species as equal to 1. Larval and nymphal instars are abbreviated as L and N, respectively, prepupa is indicated as PreP. Data on Kr-h1 as in Fig. 2, those on E93 expression of *B. germanica* are as in Fig. 3A, and those of *D. melanogaster* (E93A and E93B) and *T. castaneum* are from Ureña et al. (2014). B. Model JH pathways representing important players triggering last nymph formation at the time of the 20E peak in late penultimate nymphal instar (left) and for determining the adult fate at the beginning of the last nymphal instar (right) in a hemimetabolan insect. C: Equivalent models for triggering pupal formation at the time of the last 20E peak in prepupal stage (left) and determining adult fate at the beginning of the pupal instar (right) in a holometabolan insect. Black and grey lines represent interactions occurring or not occurring, respectively, in a given pathway; all interactions have been empirically assessed except the repression of BR-C and Kr-h1 over E93 in C, which are predicted from indirect data from functional experiments as explained in the text; in B, BR-C stands as a side branch of E93 repression because BR-C do not determine the nymphal state in hemimetabolan species.

endocrinologists (Konopova et al., 2011), further arguing for the structural and functional conservation of the Met - Kr-h1 axis in the post-embryonic development of hemimetabolan and holometabolan species. Nonetheless, despite this proposed homology, it may be useful to keep the term “nymph” for the juvenile stages of hemimetabolan species and that of “larvae” for holometabolans as these terms contain morphological and life cycle information, and also because they allow us to identify whether we are speaking about hemimetabolan or holometabolan juvenile stages without further context and explanations.

The homology of the pupa with the last nymphal instar appears to be more parsimonious than the alternative hypothesis considering that the entire set of all nymphal stages of the hemimetabolan species would be homologous to the holometabolan pupa, which was elegantly formalized by Truman and Riddiford in 1999. The Truman and Riddiford hypothesis on the origin and evolution of insect metamorphosis, which has inspired a huge amount of ideas, work and results on the subject in different laboratories, mainly builds on morphological and endocrine data (Riddiford, 2008; Truman and Riddiford, 1999, 2002, 2007). From a morphological point of view, a relevant piece of the reasoning is the definition of a pronymphal stage, exclusive of ametabolan and hemimetabolan insects, which would span the period between just before and just after eclosion from the egg (Truman and Riddiford, 1999). This is relevant for the proposal that the pupal stage is homologous to all of the nymphal instars, because the hemimetabolan pronymph would be homologous to the set of larval instars of holometabolan species (Truman and Riddiford, 1999). However, the occurrence of a pronymphal stage as defined by Truman and Riddiford has been challenged by the observations on embryo development in hemimetabolan and holometabolan species, which suggest that eclosion occurs in a homologous stage of development in all cases (Konopova and Zrzavy, 2005).

From an endocrine point of view, the comparison of the expression patterns of BR-C in hemimetabolan and holometabolan species appears to support the homology between the entire set of hemimetabolan nymphs and the holometabolan pupa, as suggested by Erezyilmaz et al. (2006). Indeed, the BR-C expression which occurs in all nymphal stages of the hemimetabolan life cycle is restricted to the prepupal stage and beginning of the pupal instar of holometabolan species (Fig. 1). Nevertheless, the comparison of expression patterns and functions in hemimetabolan and holometabolan species may alternatively suggest that BR-C proteins have been co-opted to play the complex morphogenetic functions

of pupal specifiers in the evolution from hemimetaboly to holometaboly, as argued by Huang et al. (2013). According to these authors, two key innovations affecting BR-C in post-embryonic development parallelled this evolutionary transition. The first was a shift in JH action from stimulatory (as in present hemimetabolan species) to inhibitory (present holometabolans) of BR-C expression during young stages. Thus, BR-C expression became inhibited in pre-final larval instars from holometabolan ancestors, with the resulting suppression of growth and development of BR-C-dependent tissues like the wing primordia. The second major innovation was a profound change in functions, from one specialized to direct wing development, as in present hemimetabolan species, to a wider array of morphogenetic functions, culminating in the role of triggering the pupal stage (and repressing adult morphogenesis), as seen in present holometabolan species (Huang et al., 2013). The repressor effect of E93 on BR-C expression in *T. castaneum* and *D. melanogaster* (Ureña et al., 2014) is interesting because it adds a mechanism to ensure a correct transition from pupae to adult.

Even more interesting is the fact that the repressor properties of E93 upon BR-C also operate in *B. germanica* (Ureña et al., 2014), thereby suggesting that the repressor properties of E93 upon BR-C may have been an important preadaptation of hemimetabolan species for holometabolan metamorphosis. The decrease of BR-C expression observed in the last nymphal instar of *B. germanica* (Huang et al., 2013) may also suggest that BR-C repress adult morphogenesis also in hemimetabolan insects, which would give functional sense to the aforementioned repressor properties of E93 upon BR-C in this species. Misexpression of BR-C in the last nymphal instar of *B. germanica* would give answers to this issue, but this experiment is not yet feasible in our cockroach model.

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