Upregulation of E93 Gene Expression Acts as the Trigger for Metamorphosis Independently of the Threshold Size in the Beetle Tribolium castaneum

**Highlights**

- E93 is the critical temporal factor that triggers metamorphosis in *T. castaneum*
- Upregulation of E93 correlates with attainment of the TS checkpoint
- Precocious TcE93 upregulation triggers premature metamorphosis independently of TS
- Kr-h1 represses E93 to prevent metamorphosis between mid- and late larval development

**Authors**

Silvia Chafino, Enric Ureña, Jordi Casanova, Elena Casacuberta, Xavier Franch-Marro, David Martín

**Correspondence**

xavier.franch@ibe.upf-csic.es (X.F.-M.), david.martin@ibe.upf-csic.es (D.M.)

**In Brief**

In the present study, Chafino et al. identify the upregulation of E93 expression as the trigger for metamorphosis in the holometabolous insect *Tribolium castaneum*. Depletion of E93 prevents pupa formation, whereas precocious E93 upregulation, by either JH depletion or nutrition restriction, induces premature onset of metamorphosis.
Upregulation of E93 Gene Expression Acts as the Trigger for Metamorphosis Independently of the Threshold Size in the Beetle Tribolium castaneum

Silvia Chafino,1 Enric Ureña,1 Jordi Casanova,2,3 Elena Casacuberta,1 Xavier Franch-Marro,1,4,* and David Martín1,4,*

1Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37–49, 08003 Barcelona, Spain
2Institut de Recerca Biomèdica de Barcelona (IRB Barcelona), The Barcelona Institute of Science and Technology (BIST), Baldiri Reixac, 10, 08028 Barcelona, Spain
3Institut de Biologia Molecular de Barcelona (CSIC), Baldiri Reixac, 4, 08028 Barcelona, Spain
4Lead Contact
*Correspondence: xavier.franch@ibe.upf-csic.es (X.F.-M.), david.martin@ibe.upf-csic.es (D.M.)

SUMMARY

Body size in holometabolous insects is determined by the size at which the juvenile larva undergoes metamorphosis to the pupal stage. To undergo larva-pupa transition, larva must reach a critical developmental checkpoint, the threshold size (TS); however, the molecular mechanisms through which the TS cues this transition remain to be fully characterized. Here, we use the flour beetle Tribolium castaneum to characterize the molecular mechanisms underlying entry into metamorphosis. We found that T. castaneum reaches a TS at the beginning of the last larval instar, which is associated with the downregulation of TcKr-h1 and the upregulation of TcE93 and TcBr-C. Unexpectedly, we found that while there is an association between TS and TcE93 upregulation, it is the latter that constitutes the molecular trigger for metamorphosis initiation. In light of our results, we evaluate the interactions that control the larva-pupa transition and suggest alternative models.

INTRODUCTION

Body size control is of paramount importance for the generation of viable adult organisms. In most animals, growth takes place during the immature juvenile period, and the final size of the body is fixed at adulthood (Gokhale and Shingleton, 2015). In addition to the specific genetic background, body size is the consequence of growth rate and the duration of the growth period (Gokhale and Shingleton, 2015). Although both parameters are intrinsically connected and mainly determined by environmental variables like nutritional input, temperature changes and population density (Callier and Nijhout, 2013; Gokhale and Shingleton, 2015), developmental control of body size can be considered regulation of when to stop growth. Therefore, understanding the molecular mechanisms underlying the timely exit of the growth period is key to elucidating the regulation of body size.

Metamorphosis is a clear paradigm of developmental transitions between stages of maturation in the life of organisms. In holometabolous insects, metamorphosis determines the end of growth, which occurs at larval stages, thus fixing the final size of the body (Gokhale and Shingleton, 2015; Nijhout and Callier, 2015; Nijhout et al., 2014). Historically, entry of metamorphosis has been associated with larvae reaching a critical size assessment, the threshold size (TS), first defined in the lepidopteran Manduca sexta (Nijhout, 1975) as the mass, or size, above which an animal is in the last larval instar. Larvae above the TS end growth and enter metamorphosis at the ensuing molt, whereas larvae below the TS undergo another larval molting. The length of the larval period depends on the combined action of two hormones, ecysteroid 20-hydroxyecdysone (hereafter referred to as ecdysone) and the sesquiterpenoid juvenile hormone (JH), synthesized by the prothoracic gland and the corpora allata, respectively (Hiruma and Kaneko, 2013; Jindra et al., 2013; Truman and Riddiford, 2002, 2007; Yamanaka et al., 2013). Periodic pulses of ecdysone in the presence of high levels of circulating JH promote larval molting, allowing continuous growth. In contrast, another pulse of ecdysone in the final larval stage, this time in the absence of JH, ends the larval growth period by triggering the metamorphic transition.

At the molecular level, ecdysone and JH regulate the expression of three transcription factor-encoding genes comprising what we have defined as the metamorphic gene network (MGN) (Ureña et al., 2016). One of them, Krüppel homolog 1 (Kr-h1), acts as an anti-metamorphic factor; JH induces Kr-h1 expression, preventing precocious metamorphosis until the last larval stage (Jindra et al., 2013; Minakuchi et al., 2009). A second gene, the Broad complex (Br-C), is induced by ecdysone in the absence of JH and thus restricted to a strong pulse during the last half of the last larval instar (Konopova and Jindra, 2008; Parthasarathy et al., 2008; Reza et al., 2004; Suzuki et al., 2008; Zhou et al., 1998; Zhou and Riddiford, 2002), where it acts as the pupal specifier (Daimon et al., 2015; Kiss et al., 1988; Konopova and Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008; Uhlrova et al., 2003; Zhou and Riddiford, 2002). Finally, the most recently described gene, E93, is induced by ecdysone and is highly expressed in the prepupal and pupal periods (Baehrrecke and Thummel, 1995; Kayukawa et al., 2017; Liu et al., 2015;
Figure 1. TS for T. castaneum Larvae Is Attained at the Onset of L7 and Correlates with Changes in the Expression of the MGN

(A) Percentage of animals fed with normal conditions that were in the last larval stage and underwent pupation at the ensuing molt after starvation at a given weight (n = 20–30 for each point). The dashed line was estimated from the weight, which corresponds to the 50% threshold for pupation after starvation and represents the TS checkpoint (1.9 mg). Larvae below the TS when starved molted to additional larval stages, while those starved above the TS underwent pupation.

(B) Growth curve of T. castaneum L6 and L7 larvae, displayed as body weight against age (n = 15–20 for each point). Larvae reach the TS checkpoint within the first 24 h after molting into L7 (red dashed line).

(C) Transcript levels of TcKr-h1, TcE93, and TcBr-C during the first three days of L7 (represented as weight of the larvae), that is, before and after reaching the TS checkpoint (red dashed lines), measured by qRT-PCR.

(D) Schematic diagram of the experimental nutritional conditions. Newly molted L7 larvae were reared on standard nutritional conditions (red bars) or starved (gray bars) before reaching the TS checkpoint (dashed line).

(E–G) Transcript levels of TcKr-h1 (E), TcE93 (F), and TcBr-C (G) in L7 larvae at the indicated stages and nutritional conditions as described in (D), measured by qRT-PCR. Transcript abundance values in (C)–(G) are normalized against the TcRpl32 transcript. Fold changes for each transcript are relative to their expression in newly molted L7 larvae, arbitrarily set to 1.

Error bars indicate the SEM (n = 5). Different letters in (E)–(G) represent groups with significant differences according to an ANOVA test (Tukey, p ≤ 0.05).

Ureña et al., 2014), where it controls the metamorphic transition from pupa to adult (Ureña et al., 2014, 2016). E93 is also required for the repression of Kr-h1 and Br-C expression during the pupal stage, ensuring the elimination of the two factors whose presence during this period is detrimental to adult differentiation (Ureña et al., 2014). Functional and genetic studies in the lepidopteran Bombyx mori have revealed that BmKr-h1 directly binds to promoter regions of BmBr-C and BmE93 genes, suppressing their expression and preventing larvae from undergoing precocious larval-pupal and larval-adult metamorphosis during preultimate larval stages (Kayukawa et al., 2016, 2017). However, it is still not clear how this genetic network is related to the TS and what role the TS might play. Since its definition, it has been suggested that at the TS, a putative factor would trigger entry into metamorphosis (Truman et al., 2006). However, despite extensive research, identification of this factor has remained elusive.

In the present study, we analyze the molecular mechanisms underlying entry into metamorphosis by Tribolium castaneum. We found that T. castaneum reaches a TS at the beginning of the last larval instar, which is associated with the downregulation of TcKr-h1 and the upregulation of TcE93 and TcBr-C. We also address the mechanism repressing and activating TcE93 at the different developmental stages. Unexpectedly, we found that while there is an association between the TS and the upregulation of TcE93, it is the latter that constitutes the molecular trigger for the initiation of T. castaneum metamorphosis. In light of these results, we evaluate the interactions that control the transitions from larva to pupa and suggest alternative models.

RESULTS

Characterization of the T. castaneum TS

To determine the TS in T. castaneum, we starved carefully staged larvae of defined weight and observed the nature of the following developmental transition. By doing this, we estimated that the pu11 strain of T. castaneum larvae reached the TS around 1.85–1.9 mg. Larvae above this weight were already fated to pupation even if starved, while larva below this weight molted to additional larval stages if starved (Figure 1A). Under our rearing conditions, pu11-T. castaneum animals reached the TS within the first 24 h after the molt into the seventh larval instar (L7) (Figure 1B), indicating that L7 is the last larval instar.

The TS Is Associated with Changes in the MGN

To understand the molecular basis underlying initiation of metamorphosis, we next examined the expression of the MGN genes in L7 larvae fated to metamorphosis (having reached the TS) and not yet fated to metamorphosis (before the TS). Upon reaching the TS, TcKr-h1 expression was strongly downregulated, while the expression of TcE93 and TcBr-C was significantly upregulated 7-fold and 2.5-fold, respectively (Figure 1C). To confirm that the changes in the MGN expression are associated with attainment of the TS, we starved L7 larvae just before reaching the TS and measured the mRNA levels of these genes 48 and 72 h later (Figure 1D). As Figure 1E shows, starvation before the TS resulted in persistently elevated levels of TcKr-h1, rather than the normal decline observed in continuously fed last instar larvae. We also found that starvation induces the upregulation of TcE93 and TcBr-C, consistent with their roles in repressing the metamorphic triggers TcKr-h1, TcE93, and TcBr-C.
laria. In line with this, TcE93 and TcBr-C levels did not increase in larvae starved before the TS (Figures 1F and 1G). Altogether, our results show that T. castaneum reaches the TS at the onset of L7 when larvae weigh 1.9 mg and is associated with stage-specific changes in the expression of the components of the MGN.

**Alleviation of TcKr-h1-Mediated TcE93 Repression Triggers Metamorphosis**

The preceding results suggest that the inability of pre-TS starved L7 larvae to undergo pupation stems from the sustained elevated levels of the anti-metamorphic TcKr-h1 factor. To test this possibility, we injected dsTcKr-h1 into the late L6 larvae (TcKr-h1i animals) and starved them once at L7 but before reaching the TS (Figure 2A). Specimens injected with dsMock were used as negative controls (Control animals). Whereas pre-TS starved Control larvae molted to a supernumerary L8 stage, pre-TS starved L7-TcKr-h1i animals initiated the metamorphic transition, even if they had not reached the TS, and entered into the prepupal stage on a normal schedule (Figure 2B). TcKr-h1i animals that pupated showed clear signs of acceleration of adult development, such as several rows of well-developed ommatidia in the compound eye, characteristic adult shape and segmentation in the antennae; typical adult double claws and differentiated segments in the legs, including the tarsal ones; and highly sclerotized elytra with the proper adult microsculpture (Figures S1A–S1D”, S1F, and S1G). In addition, TcKr-h1i animals lacked pupal features such as gin traps in the abdomen (Figures S1E and S1E). These results are consistent with our previous observations showing that the prepupal pulse of TcKr-h1 is critical to prevent direct adult differentiation (Ureña et al., 2016). Whereas TcE93 expression in the pre-TS starved Control L7 animals that eventually molted into a supernumerary larval stage remained below the levels detected in non-treated L7 larvae before reaching the TS (Figure 2C), in pre-TS starved L7-TcKr-h1i animals that metamorphose, only the expression of TcE93 was found to be upregulated above the basal levels observed in newly molted non-treated L7 larvae (Figure 2C). Altogether, these results suggest that TcE93 upregulation might be the responsible for the timely exit of larval development and the initiation of metamorphosis, irrespective of the larval size.

To address this issue, we depleted TcE93 by dsRNA injection in L6 instar larvae (TcE93i animals). All TcE93i L6 larvae animals molted to normal L7 larvae, but they failed to pupate and instead repeated the larval molt to a succeeding L8 instar (Figures 3A and 3B). Closer examination of the L8-TcE93i specimens revealed a perfect larval morphology (Figure 3B). To confirm the critical requirement of TcE93 upregulation for the initiation of metamorphosis, supernumerary L8-TcE93i larvae received a second injection of dsTcE93 to maintain the effect of the RNAi treatment; re-injected L8-TcE93i larvae were unable to pupate and continuously molted to new larval stages to reach L9 (65% of TcE93i larvae) or even L10 (35%) (Figures 3A and 3B). The weight of all supernumerary TcE93i larvae was always above the TS (Figure 3C), indicating that the TS cannot trigger metamorphosis initiation in the absence of TcE93 upregulation. We also confirmed that levels of TcKr-h1 were properly downregulated in L7-TcE93i larvae (Figure 3D), consistent with TcE93 being downstream of TcKr-h1 and indicating that the role of the decline of TcKr-h1 in pupation is mediated by its effect on TcE93 upregulation. In contrast, the strong upregulation of
that the upregulation of TcE93 is the key event that triggers metamorphosis.

**Premature Upregulation of TcE93 Induces Precocious Metamorphosis from L5 Larval Stages**

If upregulation of TcE93 is responsible for the initiation of metamorphosis, it must be thus possible to elicit premature metamorphosis by precociously upregulating TcE93 expression before the L7 stage. In line with this, genetic studies have shown that *T. castaneum* can undergo precocious metamorphosis when deprived of JH (Minakuchi et al., 2008), even before reaching the TS. Is this precocious metamorphosis associated with premature upregulation of TcE93? To address this issue, we examined the developmental consequence of JH deprivation from early larval development. To prevent JH production, we depleted JH acid methyltransferase-3 (*JHAMTi*) in newly emerged L4 instar larvae (*TcJHAMTi* animals), because this is the key rate-limiting JH biosynthesis enzyme that converts JH acid into JH (Minakuchi et al., 2008). All L4-*JHAMTi* larvae molted to normal L5 larvae, and then most underwent precocious metamorphosis after the L5 stage when their body weight was ~0.8–1 mg, well below the TS (Figures 4A and 4B). In contrast, Control larvae underwent three larval molts before initiating metamorphosis at the end of the L7 stage (Figures 4A and 4B). Premature *TcJHAMTi* pupae presented all characteristic pupal features, although they were 21% smaller (Figure 4B). These results are in agreement with a previous report (Minakuchi et al., 2008).

To analyze whether premature metamorphosis was associated with TcE93 upregulation, we then measured the expression of the MGN genes in mid-L5-*TcJHAMTi* larvae. TcKr-h1 levels were significantly reduced in L5-*TcJHAMTi* animals, and those of TcE93 and TcBr-C were strongly and prematurely upregulated 60- and 8-fold, respectively (Figure 4C). This result suggests that premature metamorphosis in L5-*JHAMTi* larvae stems from the same genetic response of the MGN after reaching the TS in wild-type L7 larvae, namely, the decline in TcKr-h1 levels and the subsequent upregulation of TcE93. To corroborate this, we depleted TcKr-h1 in newly molted L4 larvae and found that as *JHAMTi* larvae, all L4-*TcKr-h1i* larvae molted to L5 properly, initiated a precocious metamorphosis at the end of the L5 stage, and finally arrested (Figures 5A and 5B). Removal of the apolysed larval cuticle showed that the arrested *TcKr-h1i* animals had clearly initiated a precocious metamorphosis, as shown by a clear differentiation of the compound eyes, highly segmented legs, and everted wings and sclerotized elytra (Figure 5B). TcE93 levels were precociously upregulated in L5-*TcKr-h1i* larvae, while TcBr-C levels remained as in Control larvae (Figures 5D and 5E). We confirm that precocious metamorphosis in L5-*TcKr-h1i* animals depended on the premature upregulation of TcE93 by depleting TcKr-h1 and TcE93 simultaneously in L4 larvae (*TcKr-h1i + TcE93i* animals). As expected, L4-*TcKr-h1i + TcE93i* larvae molted into normal L5 and then molted to normal L6 larvae instead of undergoing precocious metamorphosis (Figure 5C). Altogether, these results indicate that metamorphosis in *T. castaneum* can be induced from L5 if TcE93 expression is precociously upregulated by JH or TcKr-h1 deprivation.

However, not all larval stages appear to be able to enter metamorphosis if deprived of JH or TcKr-h1. In particular, younger
larvae (L4) did not enter metamorphosis even when JH titer or TcKr-h1 expression was severely compromised (Figures 5F and 5G). Consistent with the preceding observations, TcE93 expression was not prematurely upregulated in L4-TcKr-h1i larvae and remained as low as in L4-Control larvae, in contrast to what is observed in L5-TcKr-h1i larvae (Figure 5H). However, TcBr-C expression was precociously upregulated in L4-TcKr-h1i larvae (Figure 5I), although this increase did not trigger metamorphosis at the ensuing molt, confirming that the upregulation of TcBr-C is not the causative event that promotes the onset of metamorphosis. Altogether, these results suggest that at these earlier larval stages, other mechanisms than just TcKr-h1 repression prevent upregulation of TcE93 expression, rendering larvae incompetent for metamorphosis.

**DISCUSSION**

**E93 as the Metamorphosis Trigger**

It has been long recognized that a decline in the levels of JH acts as a determinant for entry into metamorphosis of holometabolous insects (Riddiford, 1994). Here we show that in *T. castaneum*, the drop of JH leads to entry into metamorphosis only if it leads to upregulation of TcE93. The master role of TcE93 upregulation in triggering entry into metamorphosis is clearly shown by the following three results: (1) TcE93 depletion in larvae prevents pupation, inducing reiteration of larval development, even in the absence of TcKr-h1; (2) precocious upregulation of TcE93 by RNAi-mediated downregulation of TcKr-h1 triggers premature entry into metamorphosis; and (3) a precocious decline in JH drives premature entry into metamorphosis only at those stages at which it induces TcE93 upregulation...
and has no effect in the stages at which it does not induce TcE93 upregulation. Thus, our results lead us to reevaluate the role of E93 from the adult-specifier factor acting during the pupal period to a general metamorphosis trigger role.

**TcE93 Upregulation and the TS**

Upon identification of the role of JH decline in metamorphosis, it was found that this decline only happened once the larvae had reached a given size. It was then postulated that a critical mass or size would work as a developmental checkpoint, the TS, that reached a given size. It was then posited that a critical mass or size, not the larval size, determine whether larvae initiate or do not initiate metamorphosis.

**E93 and the Role of BR-C**

Based on the present and previous results (Ureña et al., 2016), we propose a genetic regulatory model for the progression from larva to pupa in *T. castaneum* based on the upregulation of TcE93, rather than that of TcBr-C (Figure 7). To date, expression of Br-C at the end of the last larval instar is considered the first genetic manifestation of the metamorphic transition in holometabolous insects (MacWhinnie et al., 2005; Zhou et al., 1998; Zhou and Riddiford, 2001, 2002). However, depleting TcBr-C in *T. castaneum* did not force larvae to a supernumerary larval molt, as would be expected if TcBr-C promotes pupal metamorphosis, but instead led to a timely molt, with animals showing a mix of larval, pupal, and adult features (Konopova and Jindra, 2008; Parthasarathy et al., 2008; Suzuki et al., 2008; Ureña et al., 2016). Similar results were observed in the neuropteran Chrysopa perla (Konopova and Jindra, 2008) and in the lepidopteran *B. mori*, where BmBr-C controls the pupal commitment of the epidermis, but not that of the imaginal discs or the primordia (Daimon et al., 2015; Uhlirova et al., 2003). These results lead to a
reevaluation of the role of Br-C and to the suggestion that Br-C is required for the coordination of the pupal morphology in different body parts, rather than the master factor that promotes metamorphosis (Konopova and Jindra, 2008). Our results confirm this suggestion by showing that TcE93 acts upstream of TcBr-C in the control of pupal metamorphosis. Consistent with TcBr-C being downstream of TcE93, we previously observed that the critical upregulation of TcE93 during L7 was properly detected in TcBr-C-depleted larvae (Uren˜a et al., 2016). Because TcE93 was required for TcBr-C repression during the pupal period (Uren˜a et al., 2014), it remains to elucidate the molecular mechanisms that account for the initial requirement of TcE93 for TcBR-C expression, although this difference might rely on different TcE93 expression levels or on an indirect regulatory effect.

Figure 6. Nutrition Restriction Promotes Precocious TcE93 Upregulation and Pupal Metamorphosis Independent of TS

(A) Larvae were fed with the standard diet throughout larval development (Control) or changed to 20% flour diet (20%-Flour) when larvae molted to the L5 stage, reaching the stage when the critical switch in TcE93 regulation occurs, and then left until the ensuing molts. Each bar indicates the length of the periods (mean ± SD) for each developmental stage after the molt into L5. Numbers of individuals are indicated on the left, and days after L5 molting are on the bottom.

(B) Growth in body weight of Control and 20%-flour-fed animals. All weights were measured on day 1 of each instar. Bars indicate the mean ± SD. Black dashed lines represent the weight at the TS checkpoint.

(C) Nutrition restriction after molting into L5 induces premature metamorphosis. Ventral views of (left) a 4-day-old Control pupa and a small premature 20%-Flour pupa and (right) Control and 20%-Flour adults. Scale bars, 0.5 mm.

(D) Transcript levels of TcKr-h1, TcE93, and TcBr-C were measured by qRT-PCR in Control and 20%-Flour mid-L5 larvae that would undergo premature metamorphosis at the end of L5. Transcript abundance values are normalized against the TcRpL32 transcript. Fold changes for each transcript are relative to the expression of each gene in Control larvae, arbitrarily set to 1.

Early and Late Larval Stages Are Distinctly Competent to Induce TcE93 Expression and Thus to Metamorphose

Cessation of JH secretion by the corpora allata (Nijhout, 1975; Nijhout and Williams, 1974) has been associated with entry into metamorphosis. In addition, several studies have shown that insects can prematurely metamorphose when deprived of JH. For example, B. mori larvae overexpressing a JH-degrading esterase (JHE) precociously pupated after the third larval instar (Tan et al., 2005). Transcription activator-like effector nucleases (TALEN)-mediated knockdown of JHAMT or Met1 in the silkworm, as well as dimolting (mod) mutants, which lacks the JH biosynthetic enzyme JH-epoxidase CYP15C1, also underwent precocious pupation after three or four larval instars (Daimon et al., 2012, 2015). Likewise, RNAi-mediated depletion of JHAMT3 or TcKr-h1 in T. castaneum larvae provoked a significant advancement of pupation time (Minakuchi et al., 2008, 2009). These results lead to the proposition of a competence theory, according to which JH signaling becomes required for suppression of metamorphosis only after insect juveniles have reached a stage of competence to undergo metamorphosis (Smykal et al., 2014).

Here, we have confirmed previous results (Minakuchi et al., 2008, 2009) and showed that the metamorphic competence in T. castaneum is acquired during early stages of larval development, long before reaching the TS. We defined this competence as the developmental stage at which TcE93 can be upregulated by depletion of JH or TcKr-h1. Based on our data, we propose a model for the control of the larval growth period and the transition to metamorphosis in T. castaneum that is based on two clearly differentiated periods (Figure 7). The first period spans from L1 to L4. During this period, larvae are not competent to induce the expression of TcE93 when JH or TcKr-h1 levels are...
depleted, which renders growth insensitive to the anti-metamorphic effect of JH and TcKr-h1. As a result, the larval genetic program is the default state of the animal in this period. Compromising the nutritional status of larvae during this period leads to persistent larval molting, with continuous weight loss of the larvae, and to the eventual death of the animals, with no signs of metamorphic transition. During this period, TcBr-C expression can be induced in the absence of TcKr-h1, although this increase is not able to trigger metamorphosis. This repressive effect is consistent with previous reports that show the repression of Br-C by Kr-h1 in different larval stages (Huang et al., 2011; Kayukawa et al., 2016; Smykal et al., 2014), and it indicates that competence for TcBr-C expression takes place before competence to express TcE93. The second period spans from L5 to L7. In contrast to the L1 to L4 stages, maintenance of the larval program from L5 to L7 depends on the presence of JH and TcKr-h1 to prevent premature activation of TcE93. This continuous repression allows larvae to keep growing before entering metamorphosis. The occurrence of a restricted metamorphic competent period during larval development appears to be conserved in hemimetabolous insects. In Blattella germanica and Pyrrhocoris apterus, for example, depletion of Kr-h1 in the early larval period promotes precocious metamorphosis that results in smaller adults (Konopova et al., 2011; Lozano and Bélès, 2011; Smykal et al., 2014; Ureña et al., 2016). Precocious metamorphosis in B. germanica is mediated by the premature upregulation of BgE93 only when BgKr-h1 is depleted after the preultimate nymphal instar, but not in earlier instars (Ureña et al., 2016). During this metamorphic competent period, TcBr-C is not upregulated in TcKr-h1i larvae, probably because depletion of TcKr-h1 leads to premature induction of TcE93, which in turn represses TcBr-C expression. This regulatory crosstalk effect is similar to the one observed in T. castaneum L7 larvae, in which the preupal surge of TcKr-h1 is required to repress TcE93, allowing the proper expression of TcBr-C during this period (Ureña et al., 2016).
Size and Entry into Metamorphosis: A Trigger or an Association?

In conclusion, our results show that upregulation of TcE93 is the factor that promotes metamorphosis. At the molecular level, there are two phases on the regulation of TcE93 expression: a first one at early larval stages that is not modified by the precocious downregulation of JH and a second one at the late larval stages, in which a decline of the JH produces a drop of TcKr-h1 that in turn activates TcE93. This developmental switch in larval TcE93 expression competence foresees the occurrence of a competence factor that would induce TcE93 expression from L5 or repress TcE93 expression until this larval stage. However, we still don’t know what triggers the decline of JH in normal conditions of development. Classical studies discarded the idea that the JH decline was the consequence of the larvae progressing through a fixed number of larval instars (Esperk et al., 2007; Kingsolver, 2007); instead, because the JH decline was associated with the larvae attaining a given body size, it was proposed that this TS would be sensed by the organism and would elicit the mechanism responsible for the JH decline (Nijhout, 1975). However, elucidation of this sensor mechanism has proven unsuccessful so far.

Alternatively, decline of JH and attaining a given body size might be consequences of a third element triggering both phenomena. In this regard, while ec dysone at low levels stimulates JH synthesis in B. mori, it suppresses JH synthesis at a high concentration (Hiruma and Kaneko, 2013); this is the kind of dose-response that might be expected for a factor responsible for the JH profile. In line with this, it has been shown that JH biosynthesis is prevented by ec dysone in the corpus allatum of Drosophila melanogaster (Liu et al., 2018). In addition, ec dysone levels have been related with growth (Delanoue et al., 2010; Dye et al., 2017; Herbosot et al., 2015), which then will put larval growth and JH decline under the regulation of a common factor. Ecdysone is also required for E93 expression (Baehrecke and Thummel, 1995; Kayukawa et al., 2017; Liu et al., 2015; Ureña et al., 2014), including in T. castaneum (data not shown), which may be related to inability to upregulate TcE93 in the early larval stages if ec dysone levels cannot be translated into transcriptional activation of TcE93. Finally, it is well known that ec dysone and JH production is tightly linked to nutritional availability and larval feeding, which allows it to coordinate exogenous and endogenous inputs in metamorphosis control.

In summary, our study reveals the pivotal role of TcE93 expression in triggering entry into metamorphosis and argues for a reconsideration of the conventional model of the regulation of the metamorphic transition in holometabolous insects. Further research is required to confirm this role of E93 in different holometabolous insects, as well as the mechanisms coordinating growth control, transition from non-competence to metamorphic competence, and decline in JH titer.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:


STAR METHODS

KEY RESOURCES TABLE

<table>
<thead>
<tr>
<th>REAGENT or RESOURCE</th>
<th>SOURCE</th>
<th>IDENTIFIER</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemicals, Peptides, and Recombinant Proteins</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose powder</td>
<td>Sigma Aldrich</td>
<td>435236; CAS: 9004-34-6.</td>
</tr>
<tr>
<td>DNase</td>
<td>Promega</td>
<td>M610A</td>
</tr>
<tr>
<td>Critical Commercial Assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SuperScript II Reverse Transcriptase</td>
<td>Invitrogen</td>
<td>18064022</td>
</tr>
<tr>
<td>GenElute™ Mammalian Total RNA Kit</td>
<td>Sigma</td>
<td>RTN350</td>
</tr>
<tr>
<td>Power SYBR Green PCR Mastermix</td>
<td>Biorad</td>
<td>1725121</td>
</tr>
<tr>
<td>pSTBlue-1 AccepTor™ Vector Kit</td>
<td>Novagen</td>
<td>70595</td>
</tr>
<tr>
<td>Experimental Models: Organisms/Strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tribolium castaneum enhancer-trap line pu11</td>
<td>Yoshinori Tomoyasu Laboratory</td>
<td>N/A</td>
</tr>
<tr>
<td>Oligonucleotides</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Primers for RT-qPCR analysis, see Table S1</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Primers for synthesis of dsRNA, see Table S1</td>
<td>This paper</td>
<td>N/A</td>
</tr>
<tr>
<td>Random hexamers</td>
<td>Promega</td>
<td>C118A31195916</td>
</tr>
<tr>
<td>Recombinant DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid: pSTBlue-1</td>
<td>Novagen</td>
<td>70596</td>
</tr>
<tr>
<td>Software and Algorithms</td>
<td></td>
<td></td>
</tr>
<tr>
<td>The R Project for Statistical Computing</td>
<td>Free available online</td>
<td><a href="https://www.r-project.org">https://www.r-project.org</a></td>
</tr>
<tr>
<td>Adobe Photoshop CS4 Extended</td>
<td>Adobe</td>
<td>N/A</td>
</tr>
</tbody>
</table>

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, David Martin (david.martin@ibe.upf-csic.es).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

The experimental model used in this study is red flour beetle T. castaneum enhancer-trap line pu11 (detailed in Key Resources Table). pu11 is a nubbin enhancer trap line that properly recapitulates the wild-type expression of nubbin and thus expresses enhanced yellow fluorescent protein (EYFP) in the wing and elytron discs beginning at 2.5 days of the last larval instar and continue to express EYFP in these discs throughout the rest of the last larval stage and the metamorphic period (Clark-Hachtel et al., 2013). T. castaneum were reared on organic wheat flour containing 5% nutritional yeast and maintained at 29°C in constant darkness.

METHOD DETAILS

RNA extraction and quantitative real-time reverse transcriptase polymerase chain reaction (qRT-PCR)

Total RNA was isolated with the GenElute™ Mammalian Total RNA kit (Sigma), DNase treated (Promega) and reverse transcribed with Superscript II reverse transcriptase (Invitrogen) and random hexamers (Promega) as previously described (Mané-Padrós et al., 2010). Relative transcripts levels were determined by real-time PCR (qPCR), using Power SYBR Green PCR Mastermix (Applied Biosystems). To standardize the qPCR inputs, a master mix that contained Power SYBR Green PCR Mastermix and forward and reverse primers was prepared (final concentration: 100nM/qPCR). The qPCR experiments were conducted with the same quantity of tissue equivalent input for all treatments and each sample was run in duplicate using 2 μl of cDNA per reaction. All the samples were analyzed on the iCycler IQ Real Time PCR Detection System (Bio-Rad). For each standard curve, one reference DNA sample was diluted serially. Primers sequences for qPCR analyses are detailed in Key Resources Table and Table S1.

RNA interference (RNAi)

RNAi in vivo was performed as previously described (Ureña et al., 2014, 2016). Control dsRNA consisted of a non-coding sequence from the pSTBlue-1 vector (dsControl). For the in vivo treatment, 1 μl of dsRNAs, concentrated up to 1 μg/μl, were injected into the
abdomen of larvae of the pu11 line. In case of co-injection of two dsRNAs, the same volume of each dsRNA solution was mixed and applied in a single injection. To maintain the RNAi effect during the successive larval instars, the same dose of dsRNAs was reapplied to all treated animals after molting into the ensuing larval stages. The primers used to generate templates via PCR for transcription of the dsRNAs are detailed in Key Resources Table and Table S1.

**Determination of the TS checkpoint**
To determine the TS checkpoint, *T. castaneum* pu11-larvae were grown in standard media until they were starved at different weights and left individually until the ensuing molt to determine whether they molt into another larval stage or pupate. TS is the point at which 50% of starved larvae pupate at the ensuing molt.

**Nutritional experiments**
*T. castaneum* pu11-larvae were reared in normal diet which consists in organic wheat flour containing 5% nutritional yeast. For nutritional restricted experiments, larvae raised in normal diet were changed to a 20%-flour diet after molting to L5 or L4. Nutritional restricted diet is composed by cellulose powder (detailed in Key Resources Table) containing 20% of organic wheat flour with 5% nutritional yeast. For the qPCR analysis of *TcE93*, *TcKr-h1* and *TcBr-C* in 20%-Flour L5 larvae that would undergo premature metamorphosis at the end of L5 stage, we selected 20%-Flour L5 larvae that showed strong EYFP⁺ signaling in the wing and elytron discs during mid-L5. Strong EYFP⁺ elytra and wings are good markers for larvae that would undergo the metamorphic transition at the ensuing molt.

**Microscopy and histological analysis**
*T. castaneum* dissections were carried out in Ringer’s saline and the different appendages were mounted directly in Glycerol 70%. All samples were examined with AxioImager.Z1 (ApoTome 213 System, Zeiss) microscope, and images were subsequently processed using Adobe photoshop.

**Scanning-electron microscopy**
Control and TcKr-hi animals of *T. castaneum* were carefully taken out of the larval cuticle with forceps when necessary. Then, they were fixed in 80% ethanol, and dehydrated with a series of graded ethanol solutions (90%, 95% and 100%) for 15 min in each solution, critical-point dried using CO₂, sputter-coated with gold-palladium, and observed under a Hitachi S-3500N scanning electron microscope.

**QUANTIFICATION AND STATISTICAL ANALYSIS**
The following statistical analyses were used in this study: Student’s t test for comparing two means and ANOVA with post hoc Tukey’s HSD test for multiple comparisons of parametric data. All statistical analyses were performed using R and the p value significance thresholds are specified in the figure legends. Error bars denoting standard error of the mean or confidence intervals are also indicated in relevant figure legend.