Ecdysone differentially regulates metamorphic timing relative to 20-hydroxyecdysone by antagonizing juvenile hormone in *Drosophila melanogaster*

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ABSTRACT

In insects, a steroid hormone, 20-hydroxyecdysone (20E), plays important roles in the regulation of developmental transitions by initiating signaling cascades via the ecdysone receptor (EcR). Although 20E has been well characterized as the molting hormone, its precursor ecdysone (E) has been considered to be a relatively inactive compound because it has little or no effect on classic EcR mediated responses. I found that feeding E to wild-type third instar larvae of *Drosophila melanogaster* accelerates the metamorphic timing, which results in elevation of lethality during metamorphosis and reduced body size, while 20E has only a minor effect. The addition of a juvenile hormone analog (JHA) to E impeded their precocious pupariation and thereby rescued the reduced body size. The ability of JHA impeding the effect of E was not observed in the *Methoprene-tolerant* (*Met*) and *germ-cell expressed* (*gce*) double mutant animals lacking JH signaling, indicating that antagonistic action of JH against E is transduced via a primary JH receptor, Met, or a product of its homolog, Gce. I also found that L3 larvae are susceptible to E around the time when they reach their minimum viable weight. These results indicate that E, and not just 20E, is also essential for proper regulation of developmental timing and body size. Furthermore, the precocious pupariation triggered by E is impeded by the action of JH to ensure that animals attain body size to survive metamorphosis.

KEY WORDS: Ecdysone; 20-Hydroxyecdysone; Juvenile hormone; Developmental timing; Body size; Metamorphosis

RUNNING TITLE: E has roles distinct from 20E

INTRODUCTION

Hormonal control is essential for coordination and regulation of many aspects of the developmental process in many organisms. Molting and metamorphosis are initiated by a rise in the titer of the ecdysteroids. A polyhydroxylated steroid, 20-hydroxyecdysone (20E), has been characterized as the principal molting hormone in insects (Gilbert et al., 2002). Upon the initiation of the endocrine signaling, 20E binds to the ecdysone receptor (EcR), a member of the nuclear hormone receptor superfamily, which heterodimerizes with another nuclear receptor, *Drosophila* retinoid X receptor homolog Ultraspiracle (USP) (Hill et al., 2013). This hormone-receptor complex directly activates expression of a small set of early response genes which code for transcription factors (Thummel, 1996). During the ecdysteroid biosynthetic process, ecdysone (E) is synthesized from dietary cholesterol in the prothoracic gland (PG) and secreted into the haemolymph (Gilbert and Warren, 2005). After secretion, E is hydroxylated to 20E in peripheral tissues including the epidermis, midgut, malpighian tubes and fat body but not in the nerve cord nor in the prothoracic gland cells. (Petryk et al., 2003).

Metamorphosis in holometabolous insects is coordinated by two hormones, 20E and juvenile hormone (JH) (Nijhout, 1998; Jindra et al., 2013). In many insect orders, such as the Hemiptera, Coleoptera and Lepidoptera, JH defines the nature of each developmental transition by interplaying with 20E. The scheme is that 20E secretion with a high titer of JH results in molting to the next instar stage prior to the last larval instar stage. JH titer drops during the last larval instar stage due to the cessation of JH synthesis. In Lepidoptera, there is one or more small peaks of ecdysteroids in the absence of JH

following attainment of a critical weight that initiate metamorphic events such as wandering behavior. Then a high peak of ecdysteroids in the presence of JH initiates pupation (Nijhout, 1998). However, recent studies indicate that JH has relatively little influence on the progression of larval morphogenesis in *Drosophila*. Knockdown of a JH biosynthetic enzyme or genetic ablation of corpus allatum cells which produce JH allows an animal to develop into the adult or pupal stage, respectively (Liu et al., 2009; Niwa et al., 2008). JH signaling is transduced via a primary JH receptor, Methoprene-tolerant (Met), which is a member of bHLH family of transcriptional factors (Ashok et al., 1998). Met function is partially redundant with its paralog, Germ-cell expressed (Gce) in *Drosophila* (Baumann et al., 2010; Godlewski et al., 2006). Therefore, mutations of both *Met* and *gce* result in the nullification of JH signaling (Abdou et al., 2011). The *Met* and *gce* double mutant animals pupariate, but die at pupal head eversion, as is shown in animals whose corpus allatum cells are genetically ablated. Neither JH-deficiency nor loss of JH signaling in *Drosophila* results in a typical phenotype of JH deficiency, such as a reduced number of larval instars, as is shown in Lepidoptera and Coleoptera (Daimon et al., 2012; Konopova and Jindra, 2007; Minakuchi et al., 2008; Tan et al., 2005). This indicates that the responses of *Drosophila* to JH are quite different from those of other insects .

Final size in insects is achieved during their larval growth period, which is terminated by the onset of metamorphosis. Before a larva is committed to pupal development, there are two checkpoints for which the larva must surpass the threshold size for metamorphosis to occur. The first checkpoint is “the minimal viable weight (MVW)” which is the minimum weight needed to successfully survive metamorphosis

(Mirth and Riddiford, 2007). The second checkpoint is "the critical weight" which is the minimum weight wherein starvation can no longer delay metamorphosis (Mirth and Riddiford, 2007), i.e. physiological processes are irreversibly initiated (Stieper et al., 2008). While there is a confusion between MVW and critical weight in *Drosophila melanogaster*, because the two check points appear at the same time (Mirth et al., 2005), the timing of the activation of E synthesis in the PG corresponds to the attainment of these checkpoints (Layalle et al., 2008; Mirth et al., 2005). The interval between the attainment of critical weight and the termination of growth, "the terminal growth period (TGP) ", is marked as an important period in the determination of final body size (Shingleton et al., 2007).

While ecdysteroid signaling in insects is transduced by a heterodimer of EcR and USP nuclear receptors, prior experimental evidence in various binding assays has indicated that E shows little or no affinity to EcR (due to the absence of the 20-hydroxy moiety) (Nakagawa and Henrich, 2009). Despite an unfavorable affinity of EcR for E, evidence for possible specific roles of E has been obtained in *Drosophila* and Lepidopteran species. For example, E enhances JH sensitivity of the PG during the larval-prepupal transition in the moth *Mamestra brassicae* (Hiruma, 1982). Feeding E to the silkworm *Bombyx mori* induces ultranumerary larval ecdysis, i.e. 7 additional molts, suggesting that the sensitivity of larval epidermis to 20E is altered by E (Tanaka, 1995; Tanaka and Takeda, 1993a, b). Also, E is required for cell proliferation during optic lobe neurogenesis at the early pupal stage in the moth *Manduca sexta* (Champlin and Truman, 1998a). In addition, an orphan nuclear receptor, DHR38, is activated by several ecdysteroids, including E, suggesting the possibility of a second ecdysteroid signaling

pathway via DHR38 (Baker et al., 2003). Furthermore, several genes transcriptionally regulated by E, but not by 20E, have been identified from *Drosophila* larval organ culture using microarray technology (Beckstead et al., 2007). These results suggest the existence of other ecdysteroid signaling pathway(s) besides that mediated by 20E and the EcR/USP receptor complex.

In this paper, I report that not only 20E but also E is essential for the regulation of metamorphic timing in *Drosophila melanogaster*. I show a role for E in the determination of the onset of metamorphosis, which couples with the antagonistic effect on JH. Furthermore, I propose a model that explains the determination of developmental timing and body size as regulated by multiple hormonal interactions around the time when L3 larvae surpass a threshold of the minimum weight to survive metamorphosis.

MATERIALS AND METHODS

Drosophila strains

The transgenic line, *UAS-Grim* (McBrayer et al., 2007), was obtained from M.B. O'Connor. *Aug-Gal4* was obtained from Korge (Siegmund and Korge, 2001). Met^{27} $gce^{2.5k}$ was obtained from Wang (Abdou et al., 2011). *UAS-GFP*, Oregon-R (OreR) and y^1w^1 (*yw*) were obtained from Drosophila Genetic Resource Center at Kyoto Institute of Technology. For genetic ablation experiments, *Aug-Gal4/CyO, GFP* was crossed to *UAS-Grim/CyO, GFP*; *UAS-GFP*. Flies were cultured on standard cornmeal/yeast extract/dextrose medium.

Chemicals

E was purchased from Sigma. 20E was a gift of K. Hiruma. Methoprene was purchased from AccuStandard (New Haven, CT, USA). Each compound was purified by reverse-phase HPLC before experiments.

Preparation of instant food for developmental analyses

Instant food with or without insect hormone(s) was prepared at room temperature by the following procedure: 100 mg of instant *Drosophila* medium, formula 4-24, (Carolina Biological Supply Co., CA, USA) and 100 mg of dry yeast were thoroughly mixed with 380 μl of water and 20 μl of a solution of insect hormone, i.e. 20μl of 20, 10, 3, 1, 0.3 and 0.1 mM of ecdysteroid dissolved in ethanol was applied to prepare for 1, 0.5, 0.15, 0.05, 0.015 and 0.005 mM at the final concentration in instant food. Methoprene dissolved in acetone was suspended in water, and then the suspension was applied to instant food at 0.5, 0.15 or 0.05 mM final concentration. To prepare for a mixture of insect hormones, 10μl of 20 mM of 20E dissolved in ethanol or else methoprene dissolved in acetone was added to 10μl of 20 mM of E, and then 20μl of the solution was applied to instant food. For unsupplied control, only 20μl of ethanol was applied to instant food.

Analyses of metamorphosis

Eggs were collected and hatched larvae were reared on instant food without insect hormone until the L2 stage. Newly ecdysed L3 larvae were collected in 2 hr intervals. No more than 5 larvae were transferred to a 1.5 ml eppendorf tube containing instant food and plugged with wet paper and a sponge. Larvae were fed with instant food containing

insect hormone(s) at 0.5 mM final concentration, unless otherwise noted. After pupariation, prepupae or pupae were collected from the eppendorf tube, rinsed and dried, and then their lengths and weight were measured. To measure metamorphic timing, pupariation was scored at every 6 hr. To analyze growth rate, larval weights were measured every 12 hr during the L3 stage. Animals were individually weighed using a Mettler AE240 balance. Animals were reared under constant light at 25°C, except for ectopic expression studies at 29°C.

Determination of MVW

MVW was determined as described in Mirth et al. (2005) with minor modifications. L3 larvae (*yw*) reared on instant food were individually weighed and transferred to a well of a 24-well cell culture plate with folded wet paper. The larvae were then starved, and pupariation was scored.

Analyses of the relationship between larval size and pupal size at a time when animals were transferred to food containing E.

L3 larvae (*yw*) reared on instant food were individually weighed and transferred to food containing E at 0.15 mM final concentration. After pupation, pupae were collected from the eppendorf tube, rinsed and dried, and their weight were measured.

Quantification of the ecdysteroids of L3 larvae using an LC/MS/MS system

Newly ecdysed L3 larvae reared on instant food without insect hormone were collected in 1 hr intervals. Larvae were fed with instant food containing ecdysteroid at 0.5 mM or

186 solvent only for 2 hrs. After feeding, larvae were collected, thoroughly washed and
187 preserved in ethanol at –20^{o} C prior to processing. Whole bodies of 20 larvae were
188 extracted with ethanol (0.5 ml X 3). The extract was centrifuged and the supernatant was
189 subsequently concentrated by evaporation. The sample was purified by adsorption on a
190 Sep-Pak C18 plus cartridge (Waters, MA, USA) as described previously (Miyashita et al.,
191 2011) and dissolved in 100 µl of ethanol. Ecdysteroids were analyzed in an LC/MS/MS
192 system consisting of an Agilent 1100 HPLC system coupled to an API3000 triple
193 quadrupole mass spectrometer (AB SCIEX, CA, USA) equipped with an electrospray
194 ionization source as described previously with minor modifications (Miyashita et al.,
195 2011). Briefly, HPLC separation was performed on Poroshell 120 EC-C18 column (2.1 X
196 50mm, Agilent, CA, USA) with a 0.3 ml/min flow rate at 30^{o}C by using 0.1% aqueous
197 acetic acid (A) and acetonitrile containing 0.1% acetic acid (B) with a gradient condition
198 of 10-90% (B) for 8 min. The injection volume of the sample was 3 µl. The amounts of
199 each ecdysteroid in the bodies of the larvae were estimated using the peak areas of the
200 selected reaction monitoring (SRM) chromatogram on the basis of a calibration curve
201 constructed using the standards.
202
203 **Histological analyses**
204 For fluorescent imaging, tissues were rinsed in PBS, fixed in 4% formaldehyde in PBS
205 for 20 min, washed and mounted on glass slides (Sullivan et al., 2000). Images were
206 taken on an Olympus BX51 fluorescence microscope.
207
208 **Quantitative RT-PCR**

Ten larvae were collected and homogenized in TRIzol Reagent (GIBCO-BRL, NY, USA). Total RNA was extracted from the homogenate and purified using RNeasy (Qiagen, Crawley, UK). Reverse transcription and quantitative RT-PCR were performed as described (Ono et al., 2012). The primers for Quantitative RT-PCR are used in described previously (Niwa et al., 2008).

RESULTS

E feeding causes increased lethality and severe reduction of body size

To characterize a potential function of E in metamorphosis, I sought to examine the effects of ecdysteroids on wild-type animals. Within two hours after molting to the L3 stage, OreR larvae were fed food containing ecdysteroid or none and their lethal phase and body size were examined. E-fed animals showed a higher lethality than that of unsupplied animals, whereas no significant difference in lethality was detected between 20E-fed animals and unsupplied animals (Fig. 1A-B). Significant differences in lethality between E-fed animals and the other animals were detected during the L3-pupal transition, indicating that E application affected the metamorphic process. I also observed a dramatically reduced body size of the resulting pupae from E-fed larvae (Fig. 1C and D). By feeding instant food containing 0.5 mM of E, female length was reduced by 10% and weight was reduced by 27%, while male length was reduced by 11% and weight was reduced by 26% compared with those of unsupplied controls. The decrease in body size, pupal length and weight was observed in both female and male animals fed with instant food containing more than 0.5 mM of E (Fig. 1E and F). In contrast, 20E had a little effect on body size. There was no significant difference in female and male pupal length

and female pupal weight between 20E-fed animals and unsupplied animals. I further examined the effects of ecdysteroids on another strain, *yw*. As shown in OreR, reduction of body size was observed in *yw* animals by feeding instant food containing more than 0.05 mM of E (Fig. 2). Female length was reduced by 12% and weight was reduced by 34%, while male length was reduced by 10% and weight was reduced by 30% by feeding instant food containing 0.15 mM of E compared with those of unsupplied controls (Fig. 2A and B). In contrast to OreR, mild but significant reduction of body size was observed in *yw* by feeding 20E, as reported previously (Delanoue et al., 2010; Jin et al., 2012). Female length was reduced by 7% and weight was reduced by 18%, while male length was reduced by 9% and weight was reduced by 14% by feeding instant food containing 0.15 mM of 20E compared with those of unsupplied controls. No significant reduction of body size was observed in *yw* animals by feeding 20E at lower concentration 0.05 mM, while mild but significant reduction of weight was observed in them by feeding E at this concentration (Fig. 2C-F). In the moth *Manduca sexta*, adult eye differentiation including optic lobe neurogenesis and progression of the morphogenetic furrow can be divided into two different ecdysteroid-dependent phases (Champlin and Truman, 1998a, b). While lower concentrations of 20E stimulated cell proliferation and furrow movement, higher concentrations of 20E were found to trigger apoptosis within the optic lobe anlagen and ommatidial maturation. Therefore, I sought to see if lower concentrations of 20E accelerate metamorphosis in contrast to higher concentrations. However, no significant reduction of body size was observed in either Ore-R or *yw* animals by feeding 20E at lower concentrations (Fig. 1E, 1F, 2E and 2F). These results indicate that the effects of

ecdysteroids differ according to genetic background, but the more severe reduction of body size is exhibited by the administration of E.

E accelerates the timing of the onset of metamorphosis

An alteration in body size can be caused either by an alteration in the duration of larval feeding, or an alteration of growth rate, or else some combination of both. Thus, I measured the time period spent in the L3 stage to pupariation and growth rate during the L3 stage. I found that the duration of the L3 stage was shortened in E-fed larvae compared to those of unsupplied and 20E-fed larvae (Fig. 3A). One-half of E-fed larvae began to pupariate between 42 hr and 48 hr after L3 ecdysis whereas one-half of unsupplied larvae began to pupariate at ~54 hr after L3 ecdysis. Application of 20E had only a mild effect on the acceleration of metamorphic timing in which half of larvae began to pupariate between 48 hr and 54 hr after L3 ecdysis. I also observed that E-fed larvae grew at a significantly slower rate than did unsupplied larvae from 24- to 36- hr after L3 ecdysis (Fig. 3B). These results, taken together, indicate that E accelerates the onset of metamorphosis and reduces the growth rate, which eventually results in a reduced body size.

The feeding experiments are based on the idea that both E and 20E are similarly absorbed through the gut. To confirm the assumption, I quantified the levels of E and 20E in larvae fed either of these substrates for 2 hrs just after L2-L3 ecdysis by LC/MS/MS analyses. As expected, the levels of ingested ecdysteroid were increased in both cases, indicating that both E and 20E are effectively incorporated through the gut (Fig. 3C). The amounts of E and 20E were approximately increased by 140 pg and 220 pg after 2 hrs

277 feeding of each ecdysteroid, respectively. Interestingly, a small amount of E was detected
278 in unsupplied L3 larvae at 2-3 hr after L3 ecdysis, but the titer of E was marginal in
279 20E-fed larvae. The reduction of E titer by application of 20E was also observed in
280 *Bombyx mori* (Tanaka and Takeda, 1993a).
281
282 **A JH analog antagonizes the action of E in the determination of the onset of**
283 **metamorphosis**
284 JH acts as a "status quo" factor and inhibits several ecdysteroid-induced events during the
285 larval-prepupal transition (Jindra et al., 2013). For example, feeding a JH analog
286 pyriproxifen to L3 larvae delayed the onset of wandering and appearance of the pupal
287 specifier Broad (Riddiford, 2012; Riddiford et al., 2003). Therefore, I sought to examine
288 if JH could affect the developmental acceleration and the reduced body size caused by E
289 application. To this end, OreR L3 larvae were fed with food containing both E and a JH
290 analog, methoprene (JHA). While animals fed with both E and JHA (E+JHA) or JHA
291 alone died during pupal-adult transition, consistent with the observation that treatment of
292 the JH mimics to *Drosophila* larvae blocked their pupal or adult development (Riddiford
293 and Ashburner, 1991), I found that the body size of E+JHA-fed animals reached a level
294 similar to that of unsupplied animals (Fig. 1C and D). Both pupal length and weight of
295 female E+JHA-fed animals were not significantly different from those of unsupplied
296 animals. For males, no significant difference of pupal length was detected between
297 E+JHA-fed animals and unsupplied animals. As shown in OreR, rescue of the reduced
298 body size was observed in *yw* animals (Fig. 2A-D). Since addition of JHA to the food
299 containing E restored the reduced body size to the normal level, I examined if JHA could

also rescue the precocious onset of metamorphosis caused by feeding E. I found that E+JHA-fed larvae began to pupariate at the same time as unsupplied animals (Fig. 3A), indicating that JHA inhibits the accelerated metamorphic development caused by E, resulting the restoration of body size to a level similar to that of unsupplied animals.

E-fed animals can initiate pupariation even with a deficiency in JH

The above results suggest that endogenous JH inhibits precocious pupariation triggered by E. If so, then E administration to JH-defective larvae may disrupt larval-prepupal transition by excessive acceleration of the onset of metamorphosis. To test this idea, I examined the potential impact of E on JH-defective animals whose corpora allata (CA) cells that produce JH were genetically ablated by the ectopic expression of a cell death gene, *grim* (Liu et al., 2009; Riddiford et al., 2010). To confirm the ablation of the CA cells, I expressed *GFP* along with or without *grim* in the CA. I observed the disappearance of the GFP-labeled tissue by ectopic expression of *grim* (Fig. 4A). I further measured expression levels of a JH biosynthetic enzyme *jhamt* in L2 and wandering L3 larvae. Its expression level in L2 and wandering L3 larvae was reduced by 90% and 84%, respectively (Fig. 4B). Unsupplied CA-ablated animals showed normal viability up to pupariation, as reported previously (Liu et al., 2009). I found, however, that 69% (n = 68) of CA-ablated animals fed with E also successfully pupariated, indicating that E does not have an effect on the lethal phase of the JH-defective animals. Furthermore, I measured prepupal length, weight and time period spent in the L3 stage of pupariated animals to see if there was any defect in body size or developmental timing in these E-fed CA-ablated animals (Fig. 4C-E). Ablation of the CA cells resulted in delay of the onset of pupariation

as shown by comparison *Aug21>grim* and control strains, *Aug21-Gal4* or *UAS-grim*, and the delay can be rescued by feeding JHA (Fig. 4E), as described previously (Liu et al., 2009). E feeding further resulted in a reduced body size in both the CA-ablated animals and control strains and the reduction was restored by addition of JHA to E (Fig. 4C-D), as was shown in wild-type OreR animals. The observed reduction in both the length and weight of E-fed CA-ablated animals compared to unsupplied CA-ablated animals (prepupal length: -16%; prepupal weight: -31%) were greater than that observed in E-fed control strains, *Aug21-Gal4* and *UAS-grim*, compared to unsupplied control strains (prepupal length: -9-10%; prepupal weight: -23-24%) (Fig. 4C-D). Despite these significantly greater reductions in length and weight, the feeding of E to CA-ablated animals accelerated the timing of metamorphosis in about same manner as in E-fed control strains (Fig. 4E). It should be noted that addition of JHA to E did not completely restore the acceleration of metamorphic timing. Taken together, timing of pupariation was accelerated in E-fed CA-ablated larvae, irrespective of whether or not JHA was also given. While the greater reduction in prepupal size of E-fed CA-ablated animals relative to that of E-fed controls is likely caused by developmental defects derived from the loss of JH, the ablation of the CA cells in these animals did not affect the late larval lethality seen with feeding E. These results indicate that L3 larva cannot be affected by E until the animal has attained the ability to initiate pupariation, even with a deficiency in JH.

The ability of JH analog to antagonize the action of E was not observed in animals lacking JH signaling

In *Drosophila*, JH signaling is transduced by a primary receptor Met, but is also substituted by a product of its paralog Gce. Therefore, Met and gce double mutations result in the loss of JH signaling (Abdou et al., 2011). To see if the antagonistic action of JH against E is transduced by the JH signaling pathway via Met or Gce, I examined the effects of the administration of insect hormone(s) on prepupal body size and metamorphic timing of Met^{27} $gce^{2.5k}$ mutant animals. The mutant larvae fed with ecdysteroid developed to prepupal stage and died as shown in CA-ablated animals. While no significant difference in prepupal weight was observed between 20E-fed and unsupplied animals, a remarkable reduction in both prepupal length and weight was observed in E-fed-animals (Fig. 5A-B). Importantly, the reduction was not restored by addition of JHA to E, as no significant difference in both prepupal length and weight was observed between E-fed and E+JHA-fed animals. I also observed that the timing of pupariation in E-fed animals was accelerated, but not restored, by addition of JHA, consistent with their reduced body size (Fig. 5C). One-half of E-fed and E+JHA-fed larvae began to pupariate between 30 hr and 36 hr after L3 ecdysis whereas one-half of unsupplied larvae began to pupariate at ~42 hr after L3 ecdysis. In contrast, application of 20E had only a mild effect on the acceleration of metamorphic timing as shown in Ore-R animals. These results indicate that the antagonistic action of JH against the acceleration of metamorphic timing by E is transduced via MET/GCE receptor(s).

L3 larvae are susceptible to E administration around the time when they reach their minimum viable weight

E-fed animals showed a higher lethality than that of unsupplied animals during larval-pupal transition (Fig. 1A-B), suggesting that these L3 larvae initiate metamorphosis before they surpass the threshold size for metamorphosis, MVW. On the other hand, the surviving animals attained pupariation but their body size was markedly reduced in every case, i.e. wild-type, CA-ablation and loss of JH signaling. Taken together, these results suggest that L3 larvae are susceptible to E around the time when they reach their MVW. To determine if this is the case, I compared the average weight of E-fed wandering larvae with their MVW (Fig. 6A). The average weight of E-fed wandering larvae was 0.75 mg. Approximately one-half of them attained pupation with an average larval weight 0.79 mg. The MVW of unsupplied animals, which corresponds to the 50% threshold for pupation after starvation, was approximately 0.75 mg. These results suggest that larvae which initiate wandering before attainment of MVW result in death during larval-pupal transition, while larvae which initiate wandering when they reach their MVW result in dramatically reduced body size. If so, feeding E to larvae after they have reached MVW would immediately trigger the onset of metamorphosis and result in decreased body size. To confirm the assumption, L3 larvae reared on normal food were individually weighed and transferred to food containing E, and then their pupal weights were measured (Fig. 6B and C). If female and male larvae, the weights of which were respectively less than 1.37 mg and 1.22 mg, were transferred to food containing E, their pupal weights were mostly less than average weight of unsupplied animals, suggesting that the metamorphic timing of these animals was accelerated. It should be noted that average pupal weights (female: 0.85 ± 0.20 mg, n = 30; male: 0.72 ± 0.19 mg, n = 71, error is SD) of animals which were fed with E before attainment of MVW were

close to the average pupal weights (female: 0.81 ± 0.19 mg, n = 31; male: 0.67 ± 0.12 mg, n = 47, error is SD) of E-fed animals. These results suggest that these larvae whose weight were less than MVW continue to feed until attainment of MVW, and then initiate the onset of metamorphosis when they attain approximately the weights of their MVW (Fig. 6D).

DISCUSSION

As the progression of developmental events is systemically regulated by the coordination of various genetic cascades, perturbation of major regulators (including hormones) are expected to result in developmental abnormalities. By feeding various ecdysteroids to wild type and JH-defective larvae, I demonstrate here that E regulates developmental timing by a mechanism that is different from that of the 20E regulatory cascade. Although JH has been characterized as a "status quo" factor in many insect species including Lepidoptera, Coleoptera and Hemiptera (Jindra et al., 2013), only a few studies have reported the status quo action of JH in preventing precocious metamorphic changes by the action of 20E in *Drosophila*, e.g. caspase-dependent programmed cell death in the larval fat body (Liu et al., 2009), differentiation events in the developing optic lobe of the prepupal brain (Riddiford et al., 2010) and activation of *broad* gene expression in the fat body (Abdou et al., 2011). Here, I characterize the action of JH in preventing the onset of metamorphosis as triggered by E. Furthermore, I propose a model that explains the determination of body size as regulated by multiple hormonal interactions.

E is a potential factor that accelerates developmental timing

I found that the application of E to L3 larvae accelerates the onset of metamorphosis, resulting in dramatically reduced body size. I also found that feeding of E to wild-type larvae accelerated the timing of the L1-L2 transition (H.O., unpublished). One possible explanation for how E coordinates the developmental transition is that E itself has a potential activity in the acceleration of developmental timing. Otherwise, E alters a sensitivity of specific tissue(s) participating in the regulation of developmental timing to other hormone(s). In lepidopteran species, E shows an ability to change the sensitivity of the PG to JH (Hiruma, 1982) and the possibility that E might change the sensitivity of epidermis to 20E has been suggested (Tanaka, 1995). It is interesting that 20E showed less of an effect on developmental timing than E in both cases of wild-type and loss of JH signaling. The acceleration of developmental timing by E was also observed in *Bombyx mori* where E-fed larvae molted into the next stage earlier than did the unsupplied larvae. However, it should be noted that 20E also shortened the duration of the larval period although not to the extent of E (Tanaka, 1995; Tanaka and Takeda, 1993a, b). Thus, sensitivity of larvae to 20E is quite different between *Drosophila* and lepidopteran species. 20E agonists which show high affinity to lepidopteran EcR also trigger a precocious larval molt (Dhadialla et al., 1998; Smagghe et al., 2012), indicating that a mechanism for the acceleration of developmental timing by 20E in Lepidoptera is regulated by EcR signaling. In eye development during metamorphosis, both E and 20E stimulate optic lobe neural proliferation and progression of the morphogenetic furrow, but a much higher concentration of E than that of 20E is required in the moth *Manduca sexta*, (Champlin and Truman, 1998a, b). In contrast, the specific effect of E on *Drosophila* development suggest to us the existence of E-specific signaling. Indeed,

previous studies have suggested the existence of other ecdysteroid signaling pathway(s) besides EcR signaling. For example, an orphan nuclear receptor DHR38 has been proposed as a mediator of atypical ecdysteroid signaling (Baker et al., 2003). Several studies have shown the existence of an ecdysteroid membrane receptor that mediates a non-genomic signaling pathway (Elmogy et al., 2004; Iga et al., 2007; Srivastava et al., 2005). As further evidence for E-specific signaling pathway(s), E-inducible genes which do not respond to 20E have been reported (Beckstead et al., 2007). Thus, it is possible that the E-specific response is triggered by signaling via an orphan nuclear receptor or a putative membrane receptor.

While E-specific signaling might be involved in the regulation of developmental timing, 20E does have a variable, but small effect on *Drosophila* development. While some studies showed little or no effect on developmental timing or body size, others observed a significant effect on them by feeding 20E to normal animals (Colombani et al., 2005; Delanoue et al., 2010; Jin et al., 2012). It seems likely that these differences are caused by either different experimental conditions or different genetic backgrounds of the animals. Indeed, I observed a significant reduction of body size of *yw* animals by application of 20E in this study. One possible explanation for this weak activity of 20E is that 20E partially activates the E-specific signaling by binding to a putative receptor of the signaling or modulating the signaling pathway. Thus, a dual regulatory system involving both hypothesized E-specific signaling and classic EcR signaling is likely required to regulate metamorphic timing in *Drosophila*.

Different interactions between ecdysteroids and JH in *Drosophila* and lepidopteran species

Interaction between 20E and JH has been extensively studied in lepidopteran species. In particular, several studies have shown that 20E regulates JH synthesis both positively and negatively depending on the stage of development (Bollenbacher, 1988; Gu and Chow, 1996; Kaneko et al., 2011). Therefore, it is plausible that application of E to L3 larvae results in a modification of JH synthesis, thereby the larvae precociously initiate the onset of metamorphosis. As in the case of wild-type animals, however, application of E accelerates metamorphic timing both in cases of JH-deficiency and loss of JH signaling, indicating that the precocious pupariation caused by E occurs regardless of JH titer. It should also be noted that 20E application only slightly accelerates the onset of the metamorphosis in the case of loss of JH signaling. In contrast to *Drosophila*, feeding 20E or 20E-antagonist to allatectomized-larvae in the final instar dramatically accelerated the onset of metamorphosis in both *Bombyx mori* and *Manduca sexta* (Kamimura et al., 2003; Reynolds et al., 2009). Taken together, these results indicate that the mechanisms underlying the interactions between ecdysteroids and JH in *Drosophila* are different from those in lepidopteran species.

Metamorphic timing is determined by the antagonistic interaction of E and JH to ensure attainment of MVW

I have clarified a function of JH in the regulation of metamorphic timing during the last larval instar stage as being a status quo factor. JH prevents an L3 larva from initiating metamorphosis triggered by E until the larva attains the most appropriate size, but the

time window during which E and JH are able to regulate this metamorphic timing is restricted (Fig. 6D). If JH is required to prevent the precocious pupariation triggered by E at the early L3 stage, then feeding E to JH-defective L3 larva would likely result in a larval or prepupal lethal phenotype. However, approximately 70% of CA-ablated larvae fed with E were able to survive during metamorphosis, indicating that most of larvae are insensitive to E before they attain their MVW. Application of JHA restored the normal developmental timing and body size in E-fed CA-ablated animals as well as in wild-type larvae, suggesting that JH functions until the termination of feeding to ensure achievement of an appropriate body size. In the moth *Manduca sexta*, JH inhibits the growth of imaginal discs as well as the initiation of metamorphosis of final instar larva, both in cooperation with nutrient dependent signals. (Suzuki et al., 2013; Truman et al., 2006). Thus, JH impedes precocious development in order to ensure normal growth that leads to an appropriate body size in both Diptera and Lepidoptera, despite the difference in the regulation of developmental process during larval-prepupal transition.

While my data clearly show that application of E can affect developmental timing and body size and the antagonistic effect of JH, an active role of endogenous E and JH in regulation of developmental process has not been proved. Interestingly, a small but significant amount of E was detected in larvae at the early L3 stage (Fig. 3C). The result suggests that there are two possible mechanisms to regulate dynamics of endogenous E, i.e. there is either a delay in release of E from the PG during early L3 stage, or there is a delay in conversion of E to 20E within peripheral tissues. If the latter is the case, endogenous E released from the PG into peripheral tissues could have a detrimental activity by excessive acceleration of the onset of metamorphosis. To prevent

this activity, the antagonistic effect by JH is required to ensure achievement of an appropriate size.

An effect of 20E was also observed on the body size of *yw* animals, indicating the existence of a narrow window during which 20E is able to trigger the initiation of metamorphosis (Fig. 6D). This time window for 20E was shorter than the one for E, so apparently 20E affected body size, but to a much smaller degree than that of E. It should be noted that the body size of 20E-fed OreR animals was also reduced, although no significant difference was detected between 20E-fed and unsupplied animals. In this case, the slight reduction of body size could be the result of a very short 20E-sensitive period. Thus, a crosstalk between multiple hormone signaling pathways coordinates the onset of metamorphosis and body size.

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Figure legends

Fig. 1. E feeding causes increased lethality and severe reduction of body size, the action of which is antagonized by JHA. Numbers in parentheses in the figures represent the number of animals. (A) Survival rate of OreR animals. Larvae were fed with instant food containing insect hormone(s) at 0.5 mM final concentration. Asterisk indicates a statistically significant difference of lethality between ecdysteroid-fed and unsupplied animals. χ^2 test: $^{**}p < 0.01$; $^{*}p < 0.05$. (B) Animals raised on food containing E. Left: An animal died during transition from L3 to prepupal stage. Middle: An animal died at stage P1 of prepupa becoming brown to unaided eyes. Right: An animal died at stage P2 of prepupa before gas bubble visible in abdomen. These stages are defined as described in Ashburner et al., 2005. (C and D) Effects of administration of insect hormone(s) on body size. Larvae were fed with instant food containing insect hormone(s) at 0.5 mM final concentration. Pupal length (C) and weight (D) of OreR animals are depicted. One-way ANOVA revealed the significant effects of administration of insect hormone(s). Bars indicated by different letters were found to be statistically significant at $P < 0.01$ by post-hoc Tukey-Kramer tests. Error bars represent 95% confidence intervals. (E and F) Effects of E on body size of OreR animals at a given concentration. The horizontal axis represents the final concentration of ecdysteroid in instant food. Pupal length (E) and weight (F) of OreR animals are depicted. Points indicated by different letters (females: capital letters; males: small letters) were found to be statistically significant at $P < 0.01$ by post-hoc Tukey-Kramer tests. Error bars represent 95% confidence intervals.

Fig. 2. Effects of insect hormone(s) on *yw* animals. Numbers in parentheses in the figures represent the number of animals. (A-D) Effects by administration of insect hormone(s) on body size. Larvae were fed with instant food containing insect hormone(s) at 0.15 mM (A and B) or 0.05 mM (C and D) final concentration. Pupal length (A and C) and weight (B and D) of *yw* animals are depicted. One-way ANOVA revealed the significant effects of administration of insect hormone(s). Bars indicated by different letters were found to be statistically significant at $P < 0.01$ by post-hoc Tukey-Kramer tests. Error bars represent 95% confidence intervals. (E and F) Effects of E on body size of *yw* animals at a given concentration. The horizontal axis represents the final concentration of ecdysteroid in instant food. Pupal length (E) and weight (F) of *yw* animals are depicted. Points indicated by different letters (females: capital letters; males: small letters) were found to be statistically significant at $P < 0.01$ by post-hoc Tukey-Kramer tests. Error bars represent 95% confidence intervals.

Fig. 3. Antagonistic action of E and JHA in the determination of metamorphic timing. Numbers in parentheses in the figures represent the number of animals. Larvae were fed with instant food containing insect hormone(s) at 0.5 mM final concentration. (A) Percentage of animals that underwent pupariation at a given time after ecdysis to the L3 stage. (B) Growth rates of L3 larvae raised on food with or without E. Asterisk indicates a statistically significant difference of weight at the same time between E-fed and unsupplied animals. Student's t-test: $*p < 0.01$. Error bars represent 95% confidence intervals. (C) Ecdysteroid titers in L3 larvae fed with or without ecdysteroid (mean ± SD, n = 3). L3 larvae within 1 hr after L2-L3 ecdysis were fed with food for 2 hrs and then

immediately preserved in ethanol for extraction. The titers are depicted as picogram (pg) of E or 20E / larva.

Fig. 4. E-fed animals can initiate precocious pupariation even with a deficiency in JH. Numbers in parentheses in the figures represent the number of animals. (A) Comparison of morphology and GFP expression between CA-ablated larva (a and a') and control larva (b and b'). The red arrows indicate the CA cells. (B) Quantitative RT-PCR analysis of the transcriptional levels of a JH biosynthetic enzyme, *jhamt*, in L2 and wandering L3 larvae (mean ± SD, n = 3). Asterisk indicates a statistically significant difference of transcriptional level between CA-ablated (*Aug21>grim*) and control strains. $*p < 0.001$. (C and D) Effects of the administration of insect hormone(s) on body size. Larvae were fed with instant food containing insect hormone(s) at 0.15 mM final concentration. Prepupal length (C) and weight (D) of CA-ablated (*Aug21>grim*) and control strains are depicted. The size of *Aug21>grim* animals are a combination of both females and males, because the CA-ablated animals died at stages before their sex became distinguishable. One-way ANOVA revealed the significant effects of application of insect hormone(s). Asterisk indicates a statistically significant difference. Student's t-test: $*p < 0.01$. Error bars represent 95% confidence intervals. (E) Percentage of animals that underwent pupariation at a given time after ecdysis to the L3 stage. Larvae were fed with instant food containing insect hormone(s) at 0.15 mM final concentration.

Fig. 5. The ability of JH analog antagonizing the action of E was not observed in animals lacking JH signaling. Numbers in parentheses in the figures represent the

number of animals. Larvae were fed with instant food containing insect hormone(s) at 0.5 mM final concentration. (A and B) Effects of administration of insect hormone(s) on body size of *Met*^{27} *gce*$^{2.5k}$ mutant animals. Prepupal length (A) and weight (B) of *Met*^{27} *gce*$^{2.5k}$ mutant animals are depicted. One-way ANOVA revealed the significant effects of administration of insect hormone(s). Bars indicated by different letters were found to be statistically significant at $P < 0.01$ by post-hoc Tukey-Kramer tests. Error bars represent 95% confidence intervals. (C) Percentage of animals that underwent pupariation at a given time after ecdysis to the L3 stage.

Fig. 6. Larvae are susceptible to E around the time when they reach their minimum viable weight (A) Percentage of animals that underwent pupation after starvation at a given size (n = 21-36 for each interval). The horizontal dashed line indicates the threshold where 50% of larvae pupariated. The vertical dashed line indicates the average weight of E-fed wandering larvae (0.75 ± 0.13 mg, n = 54, error is SD). The vertical solid line indicates the average weight of E-fed wandering larvae which attained pupation (0.79 ± 0.11 mg, n = 24, error is SD). (B and C) Relationship between larval size and pupal size at the time when they were transferred to food containing E at 0.15 mM final concentration. The horizontal dashed lines indicate the average pupal weight of E-fed animals (female: 0.81 mg; male: 0.67 mg) calculated from Fig. 2B. The horizontal solid lines indicate the average pupal weight of unsupplied animals (female: 1.22 mg; male: 0.95 mg) calculated from Fig. 2B. The vertical dashed lines indicate the MVW (0.75 mg) calculated from Fig. 6A. (D) A model for the control of the onset of metamorphosis by three insect hormones. The intervals of sensitivities of L3 larva to E and 20E are

indicated. L3 larvae become sensitive to E around the time when they reach their MVW, therefore feeding E during the indicated interval triggers the onset of pupariation. During this E-sensitive interval, JH prevents metamorphosis triggered by E. L3 larvae acquire a sensitivity to 20E for a short time before the termination of feeding, therefore 20E administration has only a small effect on metamorphic timing.

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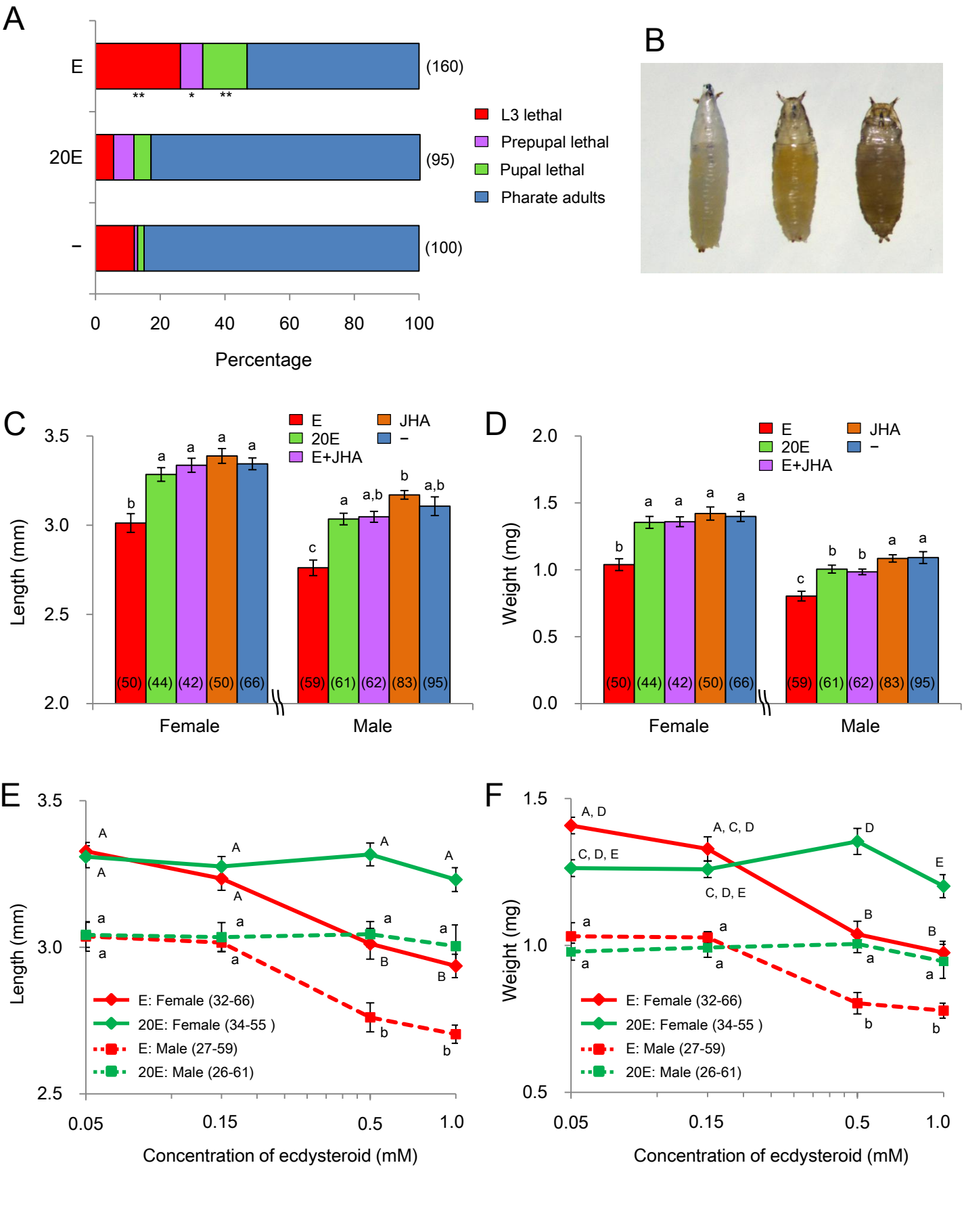
Fig. 1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Length (mm)
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E: Male (27-59)
20E: Male (26-61)
Concentration of ecdysteroid (mM)
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C, D, E
D
E
B
B
E: Female (32-66)
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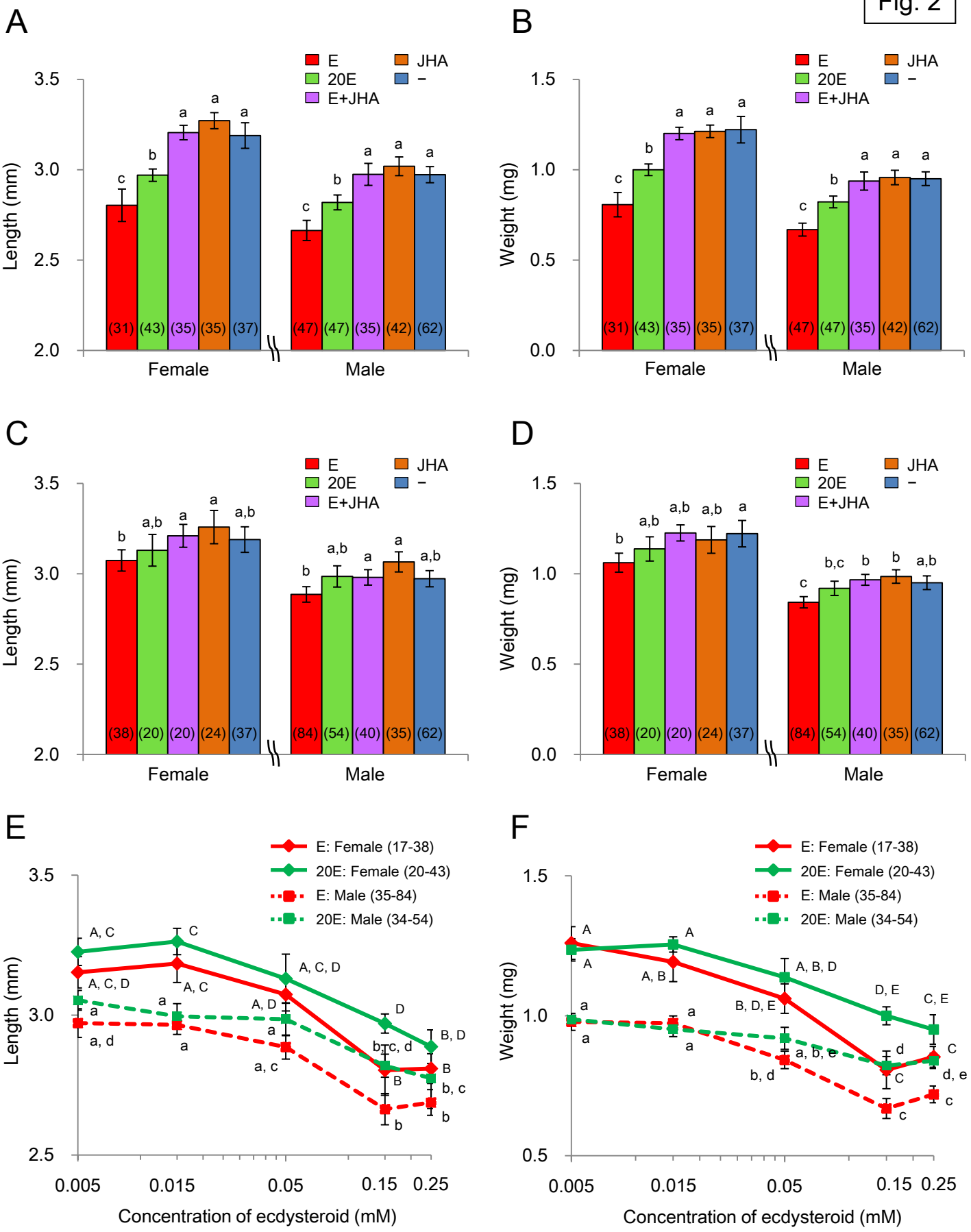
E: Male (27-59)
20E: Male (26-61)
Concentration of ecdysteroid (mM)

Fig. 2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E: Male (35-84)
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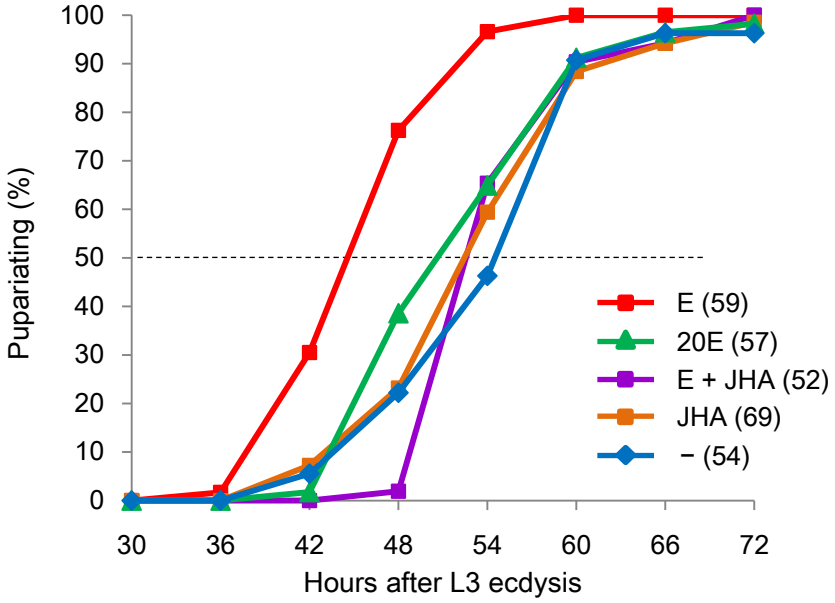
Fig. 3

A


Pupariating (%)
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36
42
48
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Hours after L3 ecdysis
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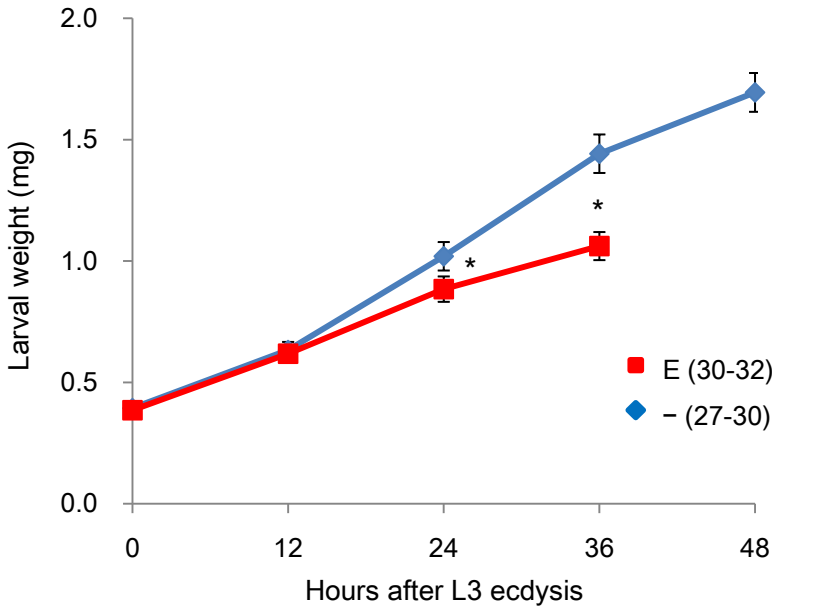
E + JHA (52)
JHA (69)
– (54)

B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Hours after L3 ecdysis
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E (30-32)
– (27-30)

C

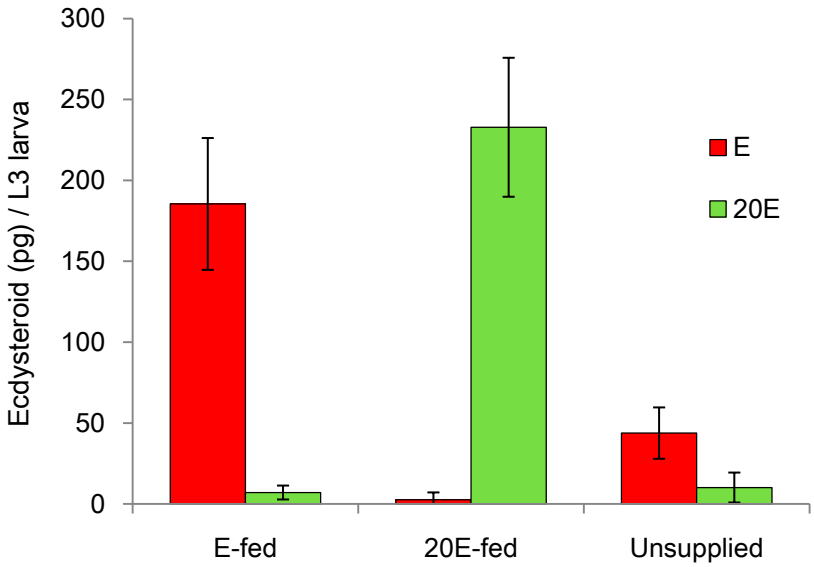

Ecdysteroid (pg) / L3 larva
300
250
200
150
100
50
0
E-fed
20E-fed
Unsupplied
E
20E

Fig. 4

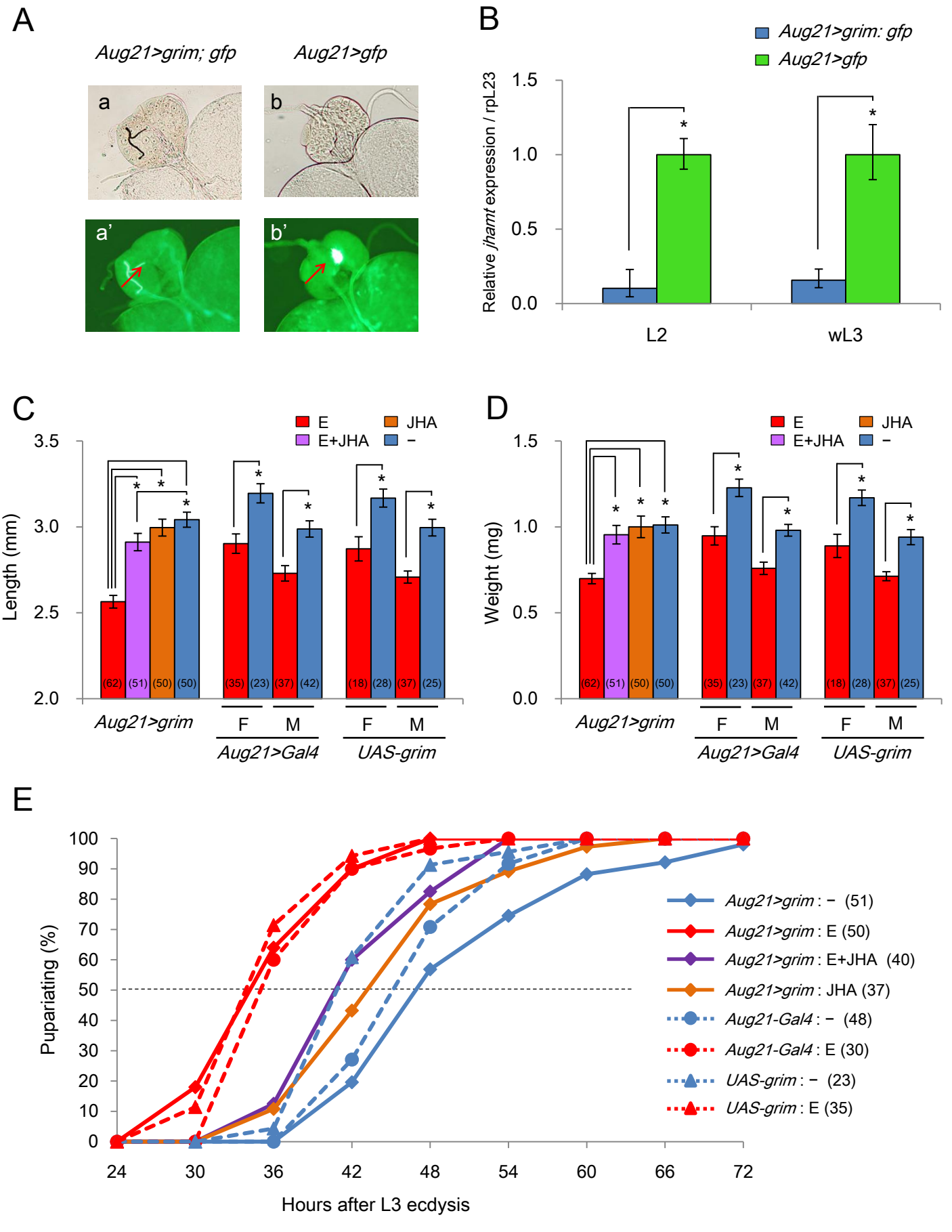

A
Aug21>grim; gfp
Aug21>gfp
a
b
a'
b'
B
Aug21>grim: gfp
Aug21>gfp
Relative jhamt expression / rpL23
1.5
1.0
0.5
0.0
L2
wL3
C
E
E+JHA
JHA
–
Length (mm)
3.5
3.0
2.5
2.0
(62) (51) (50) (50) (35) (23) (37) (42) (18) (28) (37) (25)
Aug21>grim
F M F M
Aug21>Gal4
UAS-grim
D
E
E+JHA
JHA
–
Weight (mg)
1.5
1.0
0.5
0.0
(62) (51) (50) (50) (35) (23) (37) (42) (18) (28) (37) (25)
Aug21>grim
F M F M
Aug21>Gal4
UAS-grim
E
Pupariating (%)
100
90
80
70
60
50
40
30
20
10
0
24 30 36 42 48 54 60 66 72
Hours after L3 ecdysis
Aug21>grim : – (51)
Aug21>grim : E (50)
Aug21>grim : E+JHA (40)
Aug21>grim : JHA (37)
Aug21-Gal4 : – (48)
Aug21-Gal4 : E (30)
UAS-grim : – (23)
UAS-grim : E (35)

Fig. 5

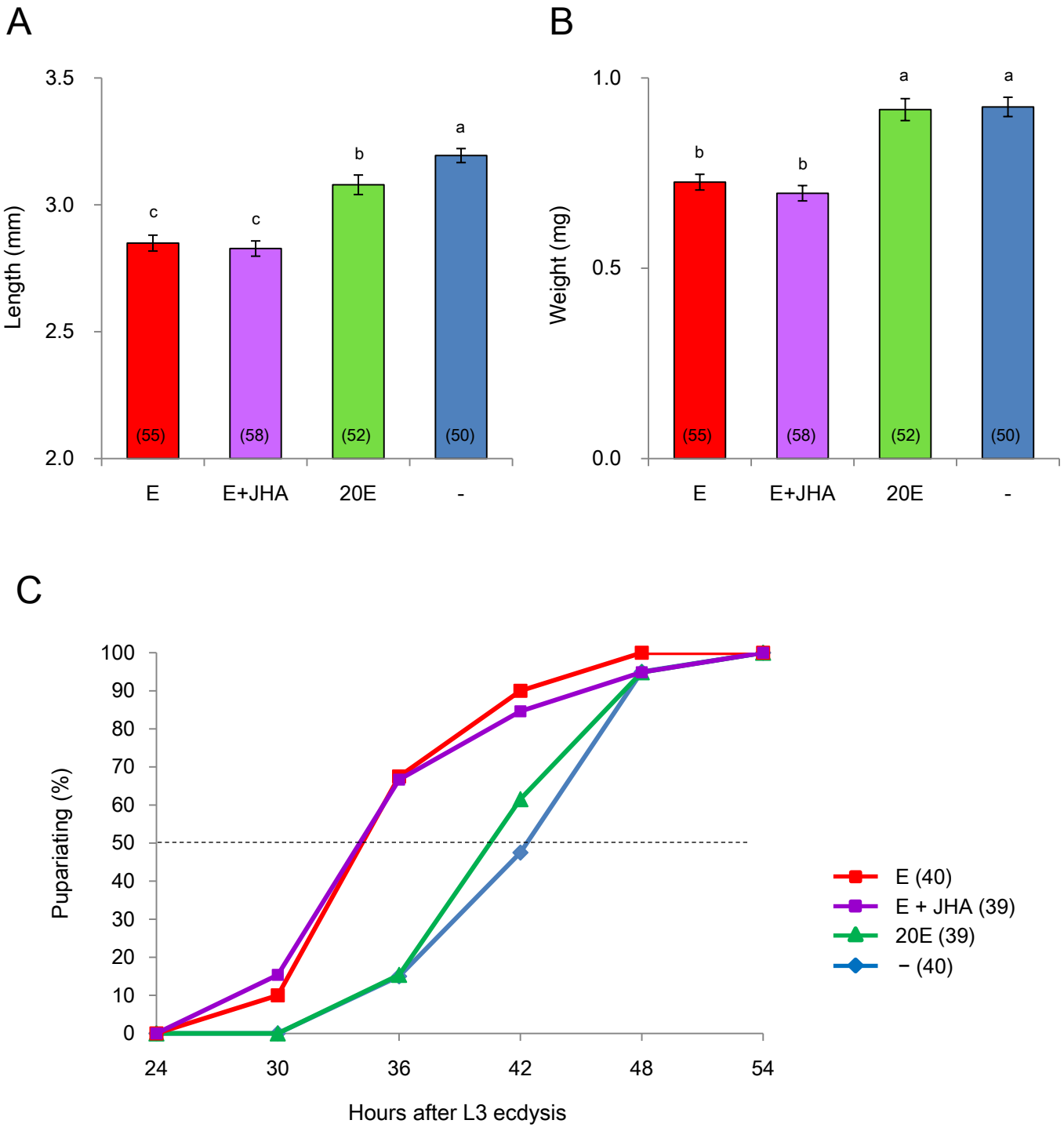
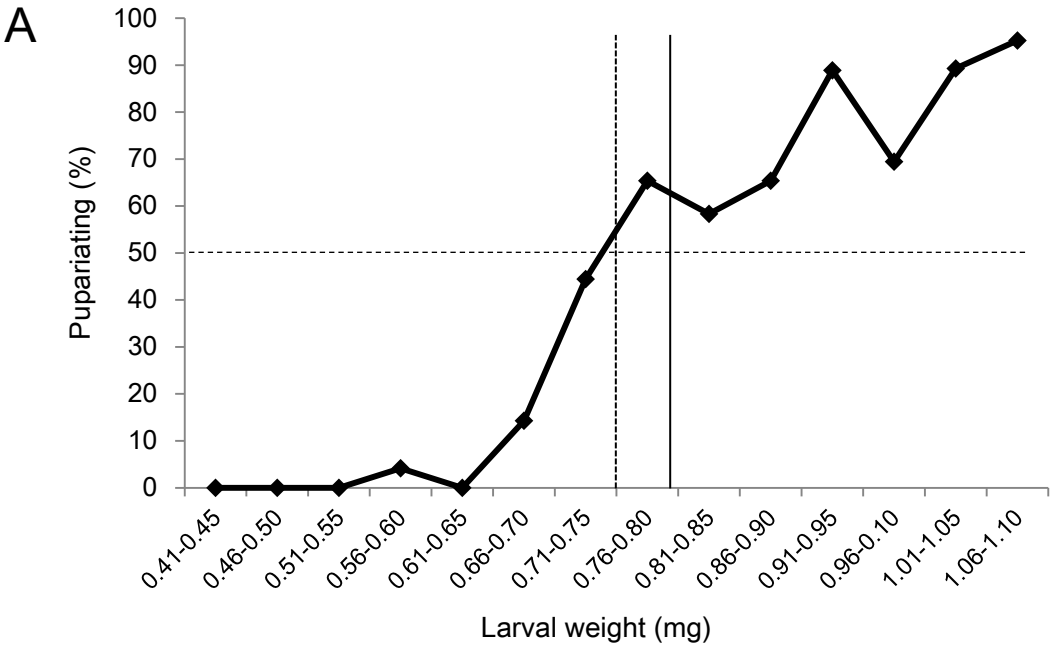
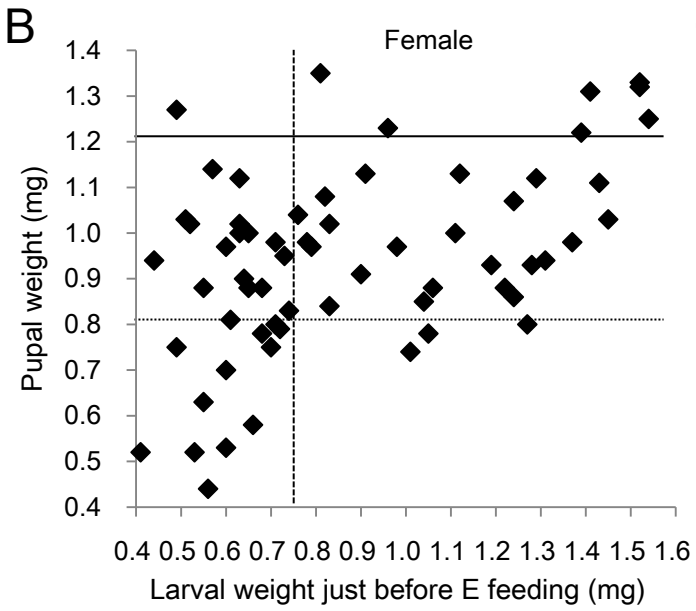
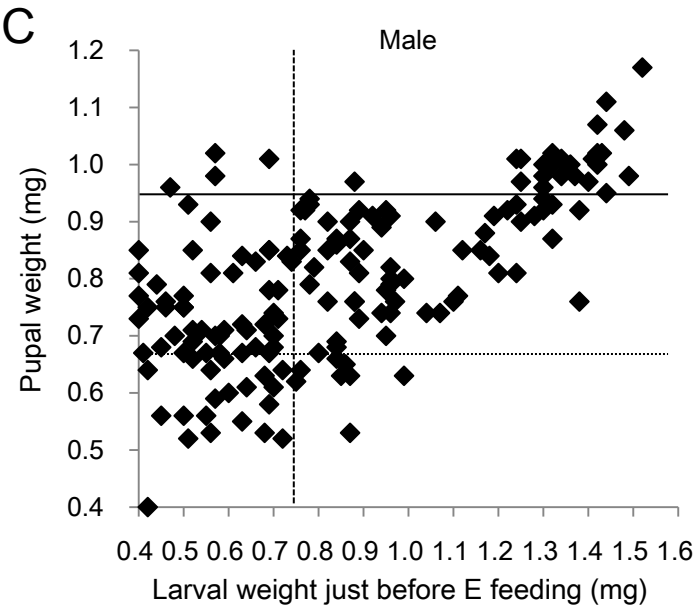
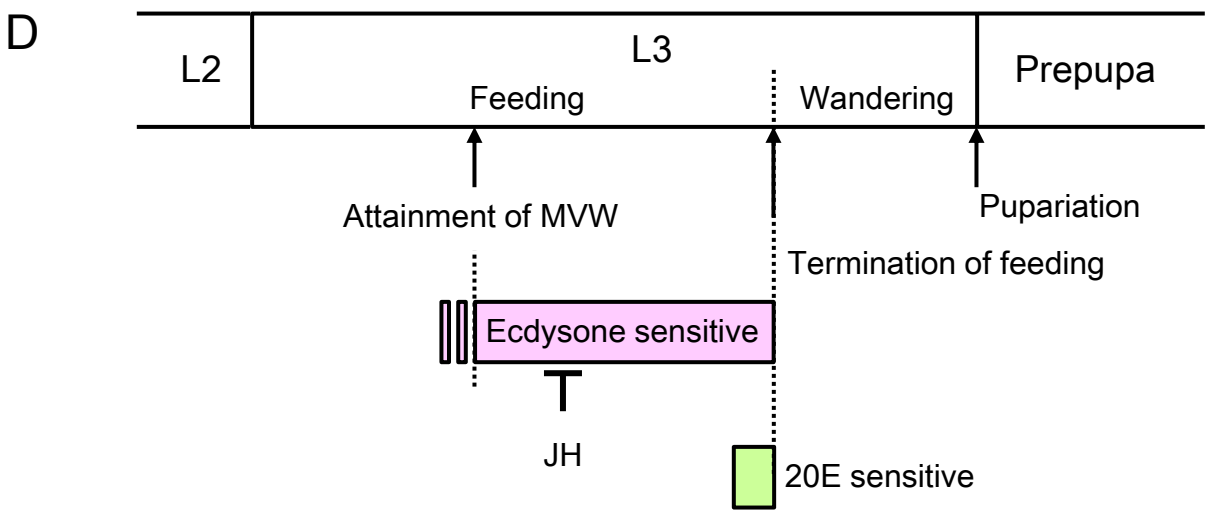

A
3.5
3.0
2.5
2.0
Length (mm)
c
c
b
a
(55)
(58)
(52)
(50)
E
E+JHA
20E
-
B
1.0
0.5
0.0
Weight (mg)
b
b
a
a
(55)
(58)
(52)
(50)
E
E+JHA
20E
-
C
100
90
80
70
60
50
40
30
20
10
0
Pupariating (%)
24
30
36
42
48
54
Hours after L3 ecdysis
E (40)
E + JHA (39)
20E (39)
− (40)

Fig. 6


A
Pupariating (%)
100
90
80
70
60
50
40
30
20
10
0
0.41-0.45
0.46-0.50
0.51-0.55
0.56-0.60
0.61-0.65
0.66-0.70
0.71-0.75
0.76-0.80
0.81-0.85
0.86-0.90
0.91-0.95
0.96-0.10
1.01-1.05
1.06-1.10
Larval weight (mg)


B
Female
Pupal weight (mg)
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6
Larval weight just before E feeding (mg)


C
Male
Pupal weight (mg)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.1 1.2 1.3 1.4 1.5 1.6
Larval weight just before E feeding (mg)


D
L2
L3
Prepupa
Feeding
Wandering
Attainment of MVW
Pupariation
Termination of feeding
Ecdysone sensitive
JH
20E sensitive