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The plant-derived triterpenoid, cucurbitacin B, but not cucurbitacin E, inhibits the developmental transition associated with ecdysone biosynthesis in *Drosophila melanogaster*

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ABSTRACT

In insects, some sterols are essential not only for cell membrane homeostasis, but for biosynthesis of the steroid hormone ecdysone. Dietary sterols are required for insect development because insects cannot synthesize sterols *de novo*. Therefore, sterol-like compounds that can compete with essential sterols are good candidates for insect growth regulators. In this study, we investigated the effects of the plant-derived triterpenoids, cucurbitacin B and E (CucB and CucE) on the development of the fruit fly, *Drosophila melanogaster*. To reduce the effects of supply with an excess of sterols contained in food, we reared *D. melanogaster* larvae on low sterol food (LSF) with or without cucurbitacins. Most larvae raised on LSF without supplementation or with CucE died at the second or third larval instar (L2 or L3) stages, whereas CucB-administered larvae mostly died without molting. The developmental arrest caused by CucB was partially rescued by ecdysone supplementation. Furthermore, we examined the effects of CucB on larval-prepupal transition by transferring larvae from LSF supplemented with cholesterol to that with CucB just after the L2/L3 molt. L3 larvae raised on LSF with CucB failed to pupariate, with a remarkable developmental delay. Ecdysone supplementation rescued the developmental delay but did not rescue the pupariation defect. Furthermore, we cultured the steroidogenic organ, the prothoracic gland (PG) of the silkworm *Bombyx mori*, with or without cucurbitacin. Ecdysone production in the PG was reduced by incubation with CucB, but not with CucE. These results suggest that CucB acts not only as an antagonist of the ecdysone receptor as previously reported, but also acts as an inhibitor of ecdysone biosynthesis.

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Key Words: Cucurbitacin B; Cucurbitacin E; Ecdysone biosynthesis; Prothoracic gland; *Drosophila melanogaster*; *Bombyx mori*

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1. Introduction

Sterols are essential not only for cell membrane homeostasis, but also for steroidogenesis in animals, including insects. Because insects lack the ability to synthesize sterols *de novo*, dietary sources of sterols are required for their normal development (Behmer and Nes, 2003; Clayton, 1964; Cooke and Sang, 1970; Hobson, 1935; Niwa and Niwa, 2011). Insects biosynthesize the steroid hormone ecdysone from dietary cholesterol in the steroidogenic organ, the prothoracic gland (PG), and subsequently secrete it into the hemolymph (Pan et al., 2021). The released ecdysone is hydroxylated to the principal molting hormone, 20-hydroxyecdysone (20E), in peripheral tissues (Lafont et al., 2012; R. Niwa and Niwa, 2014). Binding of 20E to the ecdysteroid receptor (EcR) triggers genetic cascades to fulfill various cellular processes related to molting and metamorphosis (Hill et al., 2013). Because ecdysteroids exhibit physiological activities specifically in arthropods, their biosynthesis and signaling pathways could be targets for insect growth regulators (IGRs) that do not affect the vertebrate system. Various 20E agonists, including nonsteroidal dibenzoylhydrazines, have been developed as practical IGRs (Dhadialla et al., 1998; Nakagawa, 2005). Insecticidal agonists bind to EcR with high affinities, thereby inducing premature initiation of larval molting against target insects (Wing et al., 1988).

Plant secondary metabolites are sources of IGRs owing to their huge chemical diversity, as various plant ecdysteroids and related compounds have been identified (Tarkowská and Strnad, 2016). A large number of cyclic triterpenoids and steroids are biosynthesized from squalene via cyclization (Torssell, 1983). Among them, several compounds, including plant ecdysteroids, show agonistic and/or antagonistic activities against the principal molting hormone, 20E, because their structures are similar. For

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example, plant-derived triterpenoids, cucurbitacins, have been well characterized as 20E antagonists that act on EcR (Dinan et al., 1997a, 1997b; Zou et al., 2018). Despite the antagonistic activities of cucurbitacins, outstanding growth inhibition caused by the application of cucurbitacins has not been reported (Zou et al., 2018). We presumed that supply with an excess of sterols contained in food masks the actions of cucurbitacins. Low sterol food (LSF) has been used to monitor *Drosophila melanogaster* growth to investigate the roles of sterols or steroid hormone inhibitors in previous studies (Carvalho et al., 2010; Enya et al., 2017). Here, we tested whether the analogous cucurbitacin B and E (CucB and CucE), in which structural differences are derived from the A-ring (Fig. 1A), affects *D. melanogaster* development under the restriction of sterol availability using LSF. We report that CucB, but not CucE, affects the developmental transition of *D. melanogaster* by not only antagonizing EcR, but also preventing ecdysone production.

2. Materials and methods

2.1. Insects

A wild-type strain of *D. melanogaster*, Canton-S, was obtained from the KYOTO Stock Center (DGRC) at the Kyoto Institute of Technology. Flies were cultured on a standard cornmeal/yeast extract/dextrose medium (Table S1) under constant light conditions at 25 °C. Eggs of the racial hybrid strain of *B. mori* (Kinshu × Showa) were purchased from Ueda Sanshu (Ueda, Japan). Silkworms were reared at 25 ± 1.5 °C under a 12-h light and 12-h dark photoperiod on an artificial diet “Silkmate” purchased from Nihon Nosan Kogyo (Yokohama, Japan). Most larvae started wandering on day 6 of the fifth instar.

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101 *2.2. Chemicals*

102 CucB (CAS number: 6199-67-3) was a gift that was isolated from a cucurbitaceous
103 plant (*Ceratosanthes hilariana*) by R. Nishida (Nishida et al., 1986), or purchased from
104 Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). CucE (CAS number: 18444-66-1)
105 was purchased from the Cayman Chemical Company (MI, USA). Cholesterol, 17-β
106 estradiol, and reduced glutathione were purchased from Wako Pure Chemical
107 Industries,Ltd. (Osaka, Japan). 7-Dehydrocholesterol and muristerone A were purchased
108 from Cayman Chemical Company (MI, USA). Ecdysone and 20-hydroxyecdysone were
109 purchased from AdooQ Bioscience (CA, USA) and SciTech (Prague, Czech Republic),
110 respectively. 3,4-DNADCF was synthesized as previously described (Fujikawa et al.,
111 2015).

112

113 *2.3. Developmental analysis of D. melanogaster*

114 LSF was prepared based on previous studies with some modifications (Carvalho et al.,
115 2010; Enya et al., 2017). Briefly, LSF was prepared from a mixture of 0.95 g of yeast
116 autolysate (#Y3750, Sigma–Aldrich, St. Louis, MO, USA), 1 g of glucose (Wako), 0.1
117 g of agar (Ina Food Industry, Nagano, Japan), 30 µL of propionic acid (Wako), and 30
118 µL of 10% butyl *p*-hydroxybenzoate (Nacalai Tesque, Kyoto, Japan) in 10 mL of
119 distilled water. The tested compound was dissolved in ethanol and added to the LSF at a
120 final concentration of 1 mM or 0.1 mM. For feeding rescue experiments, ecdysone
121 dissolved in ethanol was added to LSF at 0.1 mM. Canton-S eggs laid on apple juice
122 plates with yeast pastes at 25 °C overnight were collected and transferred to 200 µL of
123 food in a half-cut collection tube (2.0 mL) and plugged with a sponge. Among them,

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five or fewer hatched first-instar larvae were transferred to new food. Live larvae were transferred to new food once every two days. Dead animals were counted at each stage.

A standard cornmeal diet (440 g of yeast extract, 990 g of cornmeal, 1,100 g of D-glucose, 33 mL of propionic acid, and 38.5 mL of butyl *p*-hydroxybenzoate in 1,000 mL of distilled water) was used as normal food. For assays using normal food, Canton-S eggs were laid on grape plates with yeast pastes at 25 °C for 2 h. Hatched first-instar larvae were transferred to 1.5 ml tubes (20 larvae per tube) containing 200 µL of normal food with or without 1 mM CucB (final concentration) at 24–26 h after egg laying (AEL). Larval stages were scored by tracheal morphology every 24 h, as previously described (Enya et al., 2017).

2.4. Food ingestion assay

Twenty newly hatched larvae were transferred to LSF supplemented with or without CucB containing 1% (w/v) Brilliant Blue FCF (Wako Chemicals, Tokyo, Japan) for 12 h. Five larvae were homogenized in 5 µL H_2O using a glass microhomogenizer (#440613, 50 × 10 mm i.d., AS ONE Corporation, Osaka, Japan), and centrifuged at 2,200 × g. Absorbance of the supernatant was measured using a Nanodrop (Thermo Scientific, MA, USA) at 630 nm, which corresponds to the maximum absorbance of the Brilliant Blue FCF.

2.5. Measurement of L1 larval size

Images were captured using a Nikon SMZ645 stereomicroscope. Individual larval images were clipped using Adobe Photoshop, and the area was calculated using ImageJ (Rueden et al., 2017).

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149 *2.6. Developmental analysis of D. melanogaster animals after L2/L3 molt*

150 Canton-S eggs were placed on LSF food containing 1 mM cholesterol. After three days,
151 newly ecdysed L3 larvae within 2 h were collected and transferred to LSF containing
152 test sample(s) as prepared in section 2.3. Animal stages were counted every 24 h.
153 Larvae and prepupae were individually weighed using a microbalance (Sartorius) at the
154 indicated time after L3 ecdysis.

155

156 *2.7 Quantification of 20E by LC/MS/MS*

157 Quantification of 20E was performed as previously described (Hironaka et al., 2019;
158 Imura et al., 2020; Lavrynenko et al., 2015) with some modifications. Frozen flies were
159 individually homogenized in 200 µL of cold methanol with a pestle and centrifuged at
160 20,000 × g at 4 °C for 5 min. This procedure was repeated. Five hundred picograms of
161 muristeron A dissolved in 5 µL of methanol was added to the supernatant as an internal
162 standard. The supernatant was mixed with 100 µL of methanol, 500 µL of H_2O, and 200
163 µL of $CHCl_3$, and vortexed at room temperature for 2 min. The samples were
164 centrifuged at 20,000 × g at 4 °C for 15 min and the aqueous phase was collected and
165 dried in a vacuum concentrator, Soltrapper (Techno Sigma, Okayama, Japan). The dried
166 material was re-dissolved in 400 µL of 10% methanol. The samples were loaded on
167 MonoSpin C18 columns (GL Sciences, Tokyo, Japan) that were pre-washed with 200
168 µL of methanol and water. After sample loading and centrifugation at 3,000 × g for 1
169 min, the columns were washed with 400 µL of 10% methanol. The absorbed materials
170 were eluted with 400 µL of 60% methanol. The eluates were dried, re-dissolved in 50
171 µL of 10% methanol, and analyzed by LC-MS/MS. The LC/MS/MS system consisted

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of a Shimadzu HPLC system coupled to an API4000 triple quadrupole mass spectrometer (AB SCIEX, CA, USA) equipped with an electrospray ionization source. HPLC separation was performed on a Poroshell 120 EC-C18 column (2.1 × 50 mm, Agilent, CA, USA) at a 0.3 ml/min flow rate at 40 °C by using 0.1% aqueous formic acid (A) and acetonitrile containing 0.1% formic acid (B). The LC mobile phase was as follows: 10% (B) in (A) at 0–1 min, 10–50% (B) in (A) at 1–9 min, 50–90% (B) in (A) at 9–10 min, 90% (B) in (A) at 10–12 min; 90–10% (B) in (A) at 10–12.5 min and 10% (B) in (A) at 12.5–16 min. MS/MS analysis was performed under the following conditions: DP: 50 V; EP: 10 V; CE: 25 V CXP: 12 V. MRM transitions were as follows: 20E: *m*/*z*: 481.3 > 371.3; muristeron A: *m*/*z*: 497.3 > 297.2. The amount of 20E was calculated using the peak area of the MRM chromatogram on the basis of a standard curve obtained from serial dilutions of each standard. The values were normalized to the wet weight of the animal.

2.8. In vitro organ culture of B. mori prothoracic glands

PGs were dissected from day 7 fifth instar larvae (1 d after the onset of wandering) of *B. mori*. Replicate groups of three right or left glands were pre-cultured in basal medium (Grace's insect medium (Sigma–Aldrich) containing 0.7% bovine serum albumin, 100 units/mL penicillin, and 100 µg/mL streptomycin) at 25 °C for 30 min. Then, the glands were transferred to 200 µL of test medium containing CucB or CucE in basal medium with 0.1% Tween 80 and 2.5% ethanol. Culturing was performed at 25 °C under a 12h light and 12h dark photoperiod for two days. The opposite side of the glands were cultured without CucB or CucE as controls. After culture, 100 µL of medium was collected, dissolved in 900 µL of methanol, and vigorously stirred. After centrifugation

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at 3,000 × g for 15 min, the supernatant was transferred to a new tube, dried by evaporation, and subsequently dissolved in 100 µL of ethanol for quantification of ecdysone. The amount of ecdysone was measured using the LC/MS/MS system, as previously described. MS/MS analysis was performed under the following conditions: DP = 50 V; EP = 10 V; CE = 15 V; CXP = 12 V. Selected reaction monitoring was performed using the transition of *m*/*z* 465 > 429.

2.9. Preparation of recombinant Nobo-Dm/GSTe14

Recombinant Noppera-bo (Nobo)-Dm/GSTe14 that is essential for regulating the biosynthesis of ecdysone was prepared as described in a previous study with slight modifications (Fujikawa et al., 2015). Briefly, Nobo-Dm/GSTe14 cloned into the expression plasmid, pCOLD-III (Takara Bio, Otsu, Japan), was transformed into *Escherichia coli* BL21 (DE3) (Nippongene, Tokyo, Japan). After pre-culture at 37 °C to the midlog phase, expression of recombinant protein was induced by the addition of 1 mM IPTG and agitation at 15 °C for 16 h. The cells were collected by centrifugation (7,000 × g, 10 min, 4 °C, 10 min, 4 °C) and lysed using a French press. Cell debris were removed by centrifugation (15,000 × g, 30 min, 4 °C). Recombinant Nobo-Dm/GSTe14 was purified from the supernatant using Glutathione Sepharose 4B (GE Healthcare, IL, USA) according to the manufacturer's protocol. The purified protein was stored at − 80 °C until use.

2.10. Evaluation of inhibitory activities of cucurbitacins against Nobo-Dm/GSTe14 using 3,4-DNADCF

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We measured the fluorescence intensities of the glutathione conjugate of 3,4-DNADCF to evaluate the inhibitory activity of cucurbitacins against Nobo-Dm/GSTe14, as described in a previous paper with slight modifications (Fujikawa et al., 2015; Koiwai et al., 2021). Briefly, each reaction mixture contained 3,4-DNADCF (1μM), GSH (1 mM), Nobo-Dm/GSTe14 (25 ng/mL), and a test compound that was dissolved in 200 μL sodium phosphate buffer containing 100 mM sodium phosphate (pH 6.5), 0.005% Tween 20, and 1% DMSO. The solutions were dispensed into each well of a 96-well black polystyrene plate (#237105, Thermo Scientific, MA, USA) and fluorescence was measured using a fluorescent microplate reader, Fluoroskan Ascent FL (Thermo Scientific, MA, USA) using the following conditions: measurement type: kinetics; integration time: 300 s; lag time: 30 s; mean count: 10s; excitation: 485 nm; emission: 538 nm. Reactive activity was calculated according to the following equation: Reaction activity (%) = $(FI_{sample} - FI_{back}) / (FI_{control} - FI_{back}) \times 100$. FI_{sample}: fluorescence intensity of wells containing a test compound; $FI_{control}$: fluorescence intensity of wells without a test compound; FI_{back}: fluorescence intensity of wells without Nobo-Dm/GSTe14 and a test compound.

2.11. Statistical analysis

Statistical analyses were conducted using the R software (https://www.r-project.org/). The EC50 was calculated using a four-parameter log-logistic model of the drc extension package (Ritz et al., 2015).

3. Results

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3.1. Effects of cucurbitacins on Drosophila melanogaster development under restriction of sterol availability

To examine the effects of cucurbitacins on *D. melanogaster* development, we fed LSF to larvae to prevent the influence of excess external sterols contained in food. Most animals raised on LSF without supplementation died at the L2 or L3 stages (Fig. 1B). This growth defect was rescued by the administration of cholesterol, as reported previously (Carvalho et al., 2010; Enya et al., 2017). Strikingly, larval molting was inhibited by feeding LSF containing CucB at a final concentration of 1 mM, as L1 larvae mostly died without molting. At the lower 0.1 mM final concentration, the developmental progression of animals was less impeded, but most larvae died before or during the L2 stage. To investigate whether the severe developmental arrest was affected by food conditions, we fed larvae normal food with or without CucB. In contrast to the sterol-depleted condition, larvae raised on normal food did not exhibit any remarkable developmental arrest regardless of CucB supplementation, whereas their developmental timing was delayed, likely due to inhibition of 20E signaling, as reported previously (Zou et al., 2018) (Fig. 2A and B, Table S3).

One possible explanation for the outstanding growth defect of larvae raised on LSF containing CucB is that larvae did not ingest the provided food, because cucurbitacins have been characterized as bitter substances for animals, including some phytophagous insects (Ferguson and Metcalf, 1985; Nishida and Fukami, 1990; Zou et al., 2018). To examine whether *D. melanogaster* larvae ingested the food, newly hatched L1 larvae were fed with LSF containing blue dye supplemented with or without CucB for 12 h. Although the larvae ingested LSF containing CucB (Fig. 3A), these larvae showed a significant reduction in body size (Fig. 3B). Next, we estimated food

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consumption by measuring the maximum absorbance of the blue dye contained in larval homogenates. Food consumption by CucB-administered larvae was significantly lower than that of control larvae, which was consistent with the growth reduction (Fig. 3C). Nevertheless, an extreme decrease in food consumption was not observed in the CucB-administered larvae, therefore, we concluded that their severe developmental arrest was due to the prevention of physiological processes by ingested CucB.

The molting arrest observed in Fig. 1B is likely due to blocking of 20E signaling by CucB, which is known to antagonize 20E (Dinan et al., 1997a, 1997b; Zou et al., 2018). Therefore, we next examined whether CucE, another 20E antagonist, also inhibits animal development. Most larvae raised on LSF with CucE died during the L2 stage (Fig. 1B, Table S2). Interestingly, this growth inhibition was not as severe as CucB-administered larvae, and rather similar to animals fed unsupplemented LSF. The result that CucE has little effect on larval molting contradicts the antagonistic activity of this compound on 20E signaling. Considering that the molting arrest caused by administration of CucB was only observed in animals raised on LSF but not on normal food, the molting defect is probably not only caused by the antagonistic action of CucB on 20E signaling, but also by its other detrimental effects. Because ecdysone is biosynthesized from cholesterol, we assumed that larval molting was severely inhibited by blocking not only 20E signaling but also ecdysone biosynthesis under the deficiency of its initial precursors. To test this hypothesis, we fed larvae LSF containing both CucB and ecdysone. In contrast to the severe molting arrest of L1 larvae administered only CucB, 60% of L1 larvae initiated molting after the additional administration of ecdysone, but half died during molting from L1 and L2 (Fig. 1B: CucB + Ecd). Thus,

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supplementation with ecdysone triggered the molting of L1 larvae, but did not rescue larval development.

This phenomenon may be explained by the following possibilities. i) The antagonistic activity of CucB on 20E signaling was alleviated by excess ecdysone supplementation, thereby L1 larvae attained molting. Considering that the administration of another antagonist, CucE, allowed larvae to develop up to the prepupal stage, this hypothesis does not seem to sufficiently explain the phenomenon. ii) CucB impeded any role fulfilled by essential sterols, thereby larvae did not develop to further stage despite ecdysone supplementation. iii) CucB inhibits ecdysone biosynthesis, therefore, molting arrest was rescued by administration of ecdysone.

We validated the second possibility by feeding larvae with both essential sterols and CucB. We first confirmed that the developmental arrest due to sterol depletion was rescued by administration of 7-dehydrocholesterol and cholesterol, as reported previously (Fig. 1B, Table S2: EtOH, CLR, and 7dC) (Carvalho et al., 2010; Enya et al., 2017). We next examined whether CucB affected animal development under cholesterol or 7-dehydrocholesterol supplementation. Although animals raised on LSF containing cholesterol mostly developed into adults, additional administration of CucB impeded development and almost all animals died at larval stages (Fig. 1B, Table S2: CLR and CucB + CLR). Likewise, more than 60% of animals raised on LSF containing 7-dehydrocholesterol developed into adults, but larvae additionally administered CucB died before L3 stage without pupariation (Fig. 1B, Table S2: 7dC and CucB + 7dC). Thus, CucB inhibited developmental transitions such as larval molting and metamorphosis despite supplementation with essential sterols, suggesting that any function associated with the sterols was impaired by CucB.

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314 *3.2. Effects of cucurbitacin B on developmental transition of Drosophila melanogaster*
315 *development under restriction of sterol availability*

316 To further examine the role of CucB in the developmental transition, larvae
317 were raised on LSF containing cholesterol until L3 ecdysis, and subsequently
318 transferred to LSF containing CucB, sterol, or both. Control L3 larvae attained
319 metamorphosis (Fig. 4A), indicating that the acquired cholesterol until the L2 stage was
320 sufficient for the metamorphic process. In contrast, CucB-supplemented L3 larvae
321 mostly failed pupariation, and the remaining animals died during the pupal stage, as
322 reported previously (Zou et al., 2018) (Fig. 4A and 4B, Table S4). The larval size at the
323 late L3 stage was significantly reduced as compared with the control larvae at 48 h after
324 L3 ecdysis, although the pupariation timing was remarkably delayed (Fig. 4C and 4D).
325 Possible causes of the delay and failure of pupariation are the inhibition of growth
326 and/or ecdysone production by CucB. To examine whether ecdysone production was
327 affected by CucB administration, L3 larvae were fed with LSF containing both CucB
328 and ecdysone. Although animal developmental progression did not improve, the
329 pupariation timing was advanced to a similar time compared to the control animals (Fig.
330 4A and 4D). Next, we quantified 20E titers in individual larvae at the late L3 stage and
331 prepupae immediately after pupariation. For L3 larvae, we collected unsupplied and
332 CucB-administered L3 larvae at 48 h and 96 h after L3 ecdysis. We detected 20E in 3/8
333 L3 larvae tested in both cases, and their values were scattered (Table S5). The scattered
334 20E titers were probably caused by unsynchronized larval development on LSF food.
335 We also detected 20E in both unsupplied and CucB-administered prepupae (Fig. 4E;
336 Table S6). We did not observe a significant difference in the 20E titers per mg of

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prepupal weight between the untreated and treated groups. This result suggests that the cause of failure of pupariation by CucB administration is partially due to the delayed elevation of 20E titer, but not to deficiency of ecdysteroids.

3.3. Inhibition of ecdysone production in the PGs of Bombyx mori by cucB, but not by CucE

The feeding experiments suggest that CucB negatively affects ecdysone biosynthesis, thereby delaying the elevation of the 20E titer. To investigate whether CucB directly prevents ecdysone production in the PGs, we cultured *B. mori* PGs *in vitro*, considering the advantage of their larger size. We dissected a pair of PGs from the final instar larvae. While one side of the glands was incubated with CucB or CucE, another side of the glands was incubated without cucurbitacins as a control. We found that ecdysone titers released from the PGs were significantly reduced by incubation with CucB, but not by incubation with CucE, at 25 μg/mL (45 μM) (Fig. 5). Ecdysone titers were not affected by CucB or CucE at the lower concentration of 2.5 μg/ml (4.5 μM). These results support the idea that CucB, but not CucE, inhibits ecdysone biosynthesis in PG.

3.4. Effects of cucurbitacins on Nobo-Dm/GSTe14 activity

Ecdysone biosynthesis in the PG is achieved by many enzymatic and regulatory molecules (Danielsen et al., 2016; Iga and Kataoka, 2012; R. Niwa and Niwa, 2014; Ou et al., 2016). Among them, we were interested in examining whether the inhibitory effect of CucB on ecdysone biosynthesis was due to the inhibition of one of the ecdysteroidogenic regulatory proteins, Noppera-bo (Nobo). Nobo is a glutathione *S*-transferase (GST) that has recently been characterized as an essential protein for

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ecdysone biosynthesis in the PG (Chanut-Delalande et al., 2014; Enya et al., 2015, 2014). Nobo appears to be involved in sterol transport and/or metabolism in the PG at the early steps of ecdysone biosynthesis. We found that CucB inhibited developmental transitions such as larval molting and metamorphosis, even when supplemented with cholesterol or 7-dehydrocholesterol, which are early intermediates in ecdysone biosynthesis. Therefore, we examined whether CucB inhibits the enzymatic activity of Nobo *in vitro*. We have previously developed an easy and highly sensitive assay system to detect GST enzymatic activity using the fluorogenic substrate 3,4-DNADCF (Fujikawa et al., 2015). We mixed 3,4-DNADCF with *D. melanogaster* Nobo (also known as GSTe14) and reduced glutathione in the presence or absence of CucB, CucE, or the known inhibitor, 17β-estradiol (Fujikawa et al., 2015; Koiwai et al., 2020), at various concentrations. We then measured the fluorescence intensity of the reaction products of 3,4-DNADCF and calculated the relative activities by subtracting the background fluorescence as an indicator of GST activity. We confirmed the inhibitory activity of 17β-estradiol (EC_{50} = 2.2 μM), as reported previously (Fujikawa et al., 2015; Koiwai et al., 2020), but did not find any apparent inhibitory activity of either CucB or CucE (Fig. 6). These results imply that CucB targets other component(s) to inhibit ecdysone biosynthesis.

4. Discussion

In this study, we focused on plant-derived triterpenoids, CucB and CucE, as IGRs because of their structural similarities with sterols. Previous studies have reported the antagonistic activities of both CucB and CucE against 20E on binding to EcR (Dinan et al., 1997a, 1997b; Zou et al., 2018). Besides, using LSF to evaluate the effects of

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cucurbitacins on *D. melanogaster* development, we found that CucB, but not CucE, inhibited the developmental progression of *D. melanogaster*. Furthermore, we found that CucB, but not CucE, inhibited ecdysone production in *B. mori* PGs *in vitro*. This result supports the hypothesis that CucB directly inhibits ecdysone biosynthesis. We presume that the different inhibitory activities between CucB and CucE are probably due to the different structures in the A-ring.

When we administrated CucB to L3 larvae after L2/L3 molt, we found that the animals exhibited developmental delay and pupariation defect, as reported previously (Zou et al., 2018), and still produced detectable 20E. These data imply that the developmental delay is due to the delay in 20E titer elevation, rather than the complete inhibition of 20E production. Of note, prolonged larval periods have been observed in animals with loss of function of the neuropeptide prothoracicotropic hormone (PTTH), which stimulates the production of ecdysone in the PGs (McBrayer et al., 2007; Shimell et al., 2018). The rise of 20E titer to trigger metamorphosis is delayed by the loss of function of PTTH, thereby prolonging the larval period and increasing animal body size (McBrayer et al., 2007; Shimell et al., 2018). The similar phenotype has also been observed in the inhibition of the PTTH signaling pathway (Caldwell et al., 2005; Rewitz et al., 2009). In contrast to the increase in animal body size by loss of PTTH function (McBrayer et al., 2007; Shimell et al., 2018), larval size was reduced in CucB-administered L3 larvae. Although we did not examine whether PTTH or its downstream signaling molecules, such as Ras and MAP kinase (Smith and Rybczynski, 2012), is involved in the inhibition of ecdysone production and/or 20E elevation by CucB, the phenotypic discrepancy implies that PTTH signaling might not be involved in the defects. Rather, we surmise that there are two possible explanations to interpret the

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CucB-induced defect: (i) Growth inhibition could indirectly cause the delayed 20E elevation. (ii) CucB directly blocks ecdysone biosynthesis in the PG cells. These two possible explanations are not necessarily exclusive to each other. Therefore, CucB probably has more than one mode of action.

Because the cyclic triterpenoids derived from natural products have similar skeletons to sterols, we hypothesized that CucB inhibits Nobo enzymatic activity. However, we found significant inhibitory activity of neither CucB nor CucE. Therefore, CucB likely targets other components required for ecdysone biosynthesis. It is well known that ecdysone biosynthesis is catalyzed by many other enzymes, such as cytochrome P450 enzymes (Iga and Kataoka, 2012; R. Niwa and Niwa, 2014). Moreover, studies in the last decades have identified a number of regulatory components of ecdysone biosynthesis in the PG, such as transcription factors, signal transduction pathways, and autophagy (Niwa and Niwa, 2016; Y. S. Niwa and Niwa, 2014; Pan et al., 2021, 2019; Texada et al., 2019). Thus, various components could be targeted by CucB. It is noteworthy that the difference between CucB and CucE is the diosphenol and α-ketol structures in the A-ring, respectively. Characterization of the physicochemical properties of cucurbitacins underlying the deterrent effects on *D. melanogaster* development would provide insights into the potent targets of highly oxygenated triterpenoids on insect growth regulation. Because ecdysone is an arthropod-specific steroid, the biosynthetic pathway is a desirable target for IGRs. CucB might serve as a candidate molecule to develop a novel type of IGR that inhibits ecdysone biosynthesis.

CRediT authorship contribution statement

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Miwako Toyofuku: Investigation. **Daiki Fujinaga**: Investigation, Writing -review & editing. **Kazue Inaba**: Investigation. **Tomoki Funahashi**: Investigation. **Yuuta Fujikawa**: Investigation, Methodology, Writing -review & editing. **Hideshi Inoue**: Supervision. **Hiroshi Kataoka**: Supervision. **Ryusuke Niwa**: Funding acquisition, Investigation, Supervision, Writing -review & editing. **Hajime Ono**: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing - original draft.

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Data availability

Data underlying this study is deposited in Mendeley data (doi: 10.17632/zzp9s6bcp9.1).

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Figure Captions

Figure 1. Developmental progression of animals raised on low sterol food supplemented with or without cucurbitacins and/or sterols. (A) Chemical structures of cucurbitacin B and E. (B) Percentage of dead animals at each stage. Final concentration of supplied sterols was 1 mM, unless otherwise noted. EtOH: ethanol; Ecd: ecdysone; CLR: cholesterol; 7dC: 7-dehydrocholesterol. Numbers in parentheses represent the number of animals.

Figure 2. Survival rate and developmental progression of animals fed normal food at a given time after egg laying. Numbers in parentheses represent the number of animals. (A) Animals were raised on normal food without supplementation (control). (B) Animals were raised on normal food supplemented with cucurbitacin B (CucB).

Figure 3. Larval feeding and growth raised on low sterol food containing supplemented with or without cucurbitacin B (CucB) at 12 h after hatching. (A) Images of first instar larvae raised on dye-containing food supplemented with or without CucB. Scale bars: 0.5 mm. (B) Larval body size defined by an area of individual larval photos. Student's *t*-test: $^{**}p < 0.001$ (n = 15–17). (C) Maximum absorbance of Brilliant Blue FCF contained in larval homogenates. Welch's *t*-test: $^{*}p < 0.01$ (n = 7). (B and C) Lines indicate mean.

Figure 4. Effects of cucurbitacin B (CucB) on animals after L2/L3 molt. (A) Percentage of dead animals at each stage. (B) Upper panel: Animals raised on low sterol food (LSF) containing CucB. Lower panel: Animals (left: prepupa; right: pupa) raised on

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LSF without CucB. Scale bars: 1 mm. (C) Larval weight raised on LSF supplemented with or without CucB at a given time after L2/L3 molt. The box plot shows 25–75% (box), median (band inside), and minima to maxima (whiskers). Boxes with different letters are significantly different at $p < 0.05$ as determined by Steel-Dwass test (n = 21–39). (D) Percentage of animals that pupariated at a given time after L2/L3 molt. (E) 20E titer of individual L3 larvae raised on LSF supplemented with or without CucB at a given time after L3 ecdysis. Lines indicate mean. No significant difference was detected. (Welch's t-test: $p > 0.05$) (n = 8). (A, C, D, E) EtOH: ethanol; CLR: cholesterol Ecd: ecdysone. (A and D) Numbers in parentheses represent the number of animals.

Figure 5. Relative amount of ecdysone in culture medium released from prothoracic glands of *Bombyx mori* under application of cucurbitacin B, E, or not (control). The relative amounts of ecdysone indicate peak area ($\times 10^3$) measured using LC/MS/MS. CucB: cucurbitacin B; CucE: cucurbitacin E. Lines indicate mean. Welch's t-test: $*p < 0.01$ (n = 4–6).

Figure 6. Inhibitory activities of cucurbitacin B, E and 17β-estradiol against glutathione-conjugation activity of Noppera-bo (Nobo-Dm/GSTe14). CucB: cucurbitacin B; CucE: cucurbitacin E. 17β-estradiol was used as a positive control. Dots and error bars represent means and SE, respectively (n = 3).

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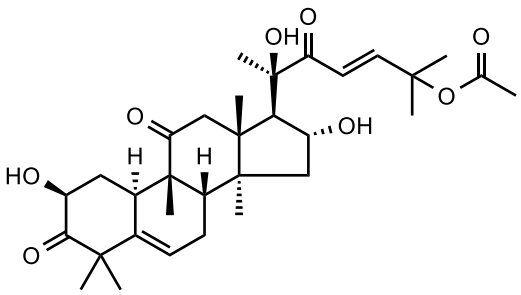
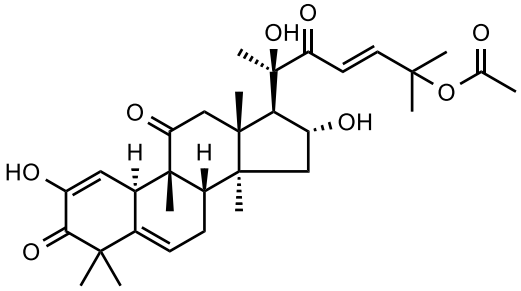
Fig. 1

A

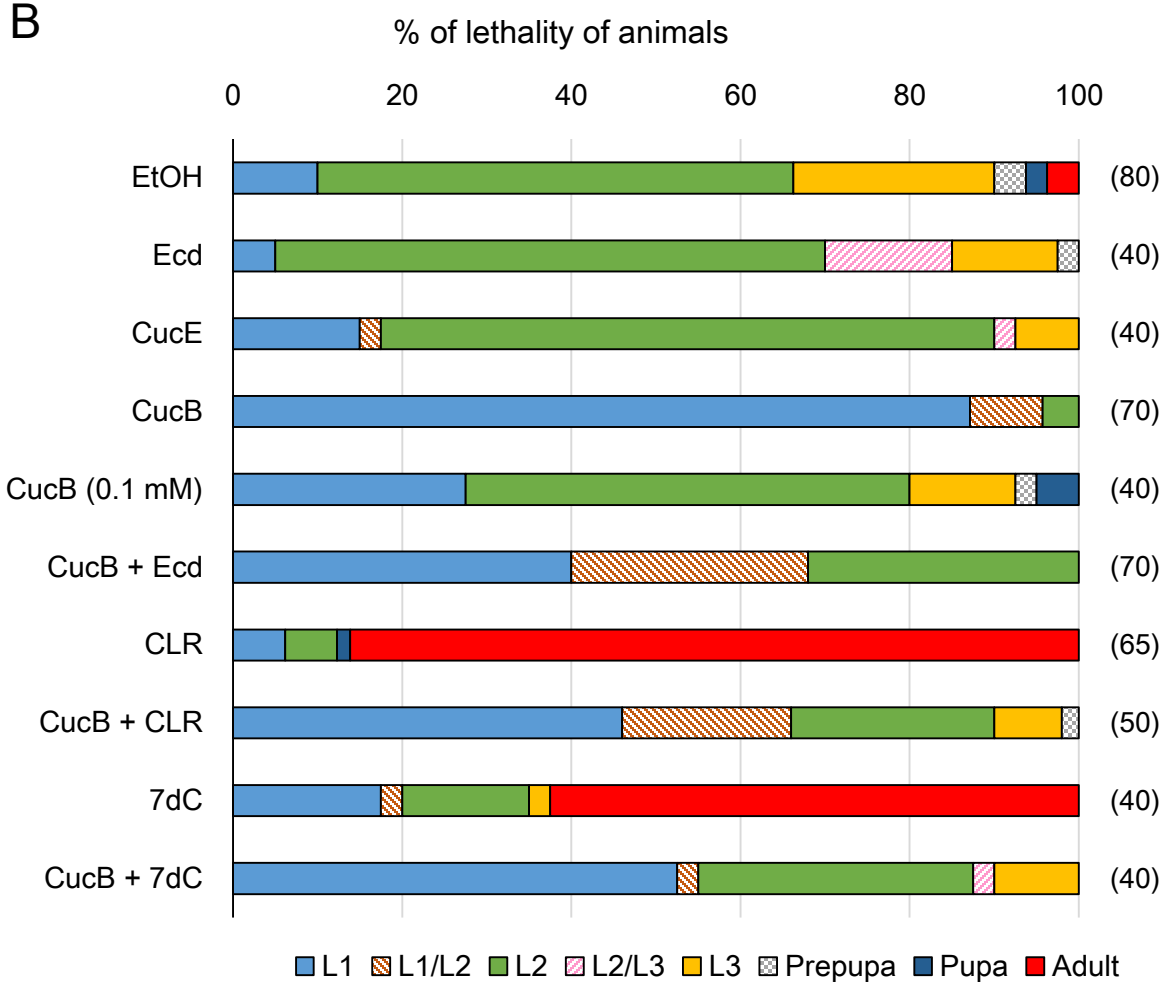


Cucurbitacin B (Cuc B)



Cucurbitacin E (Cuc E)

B



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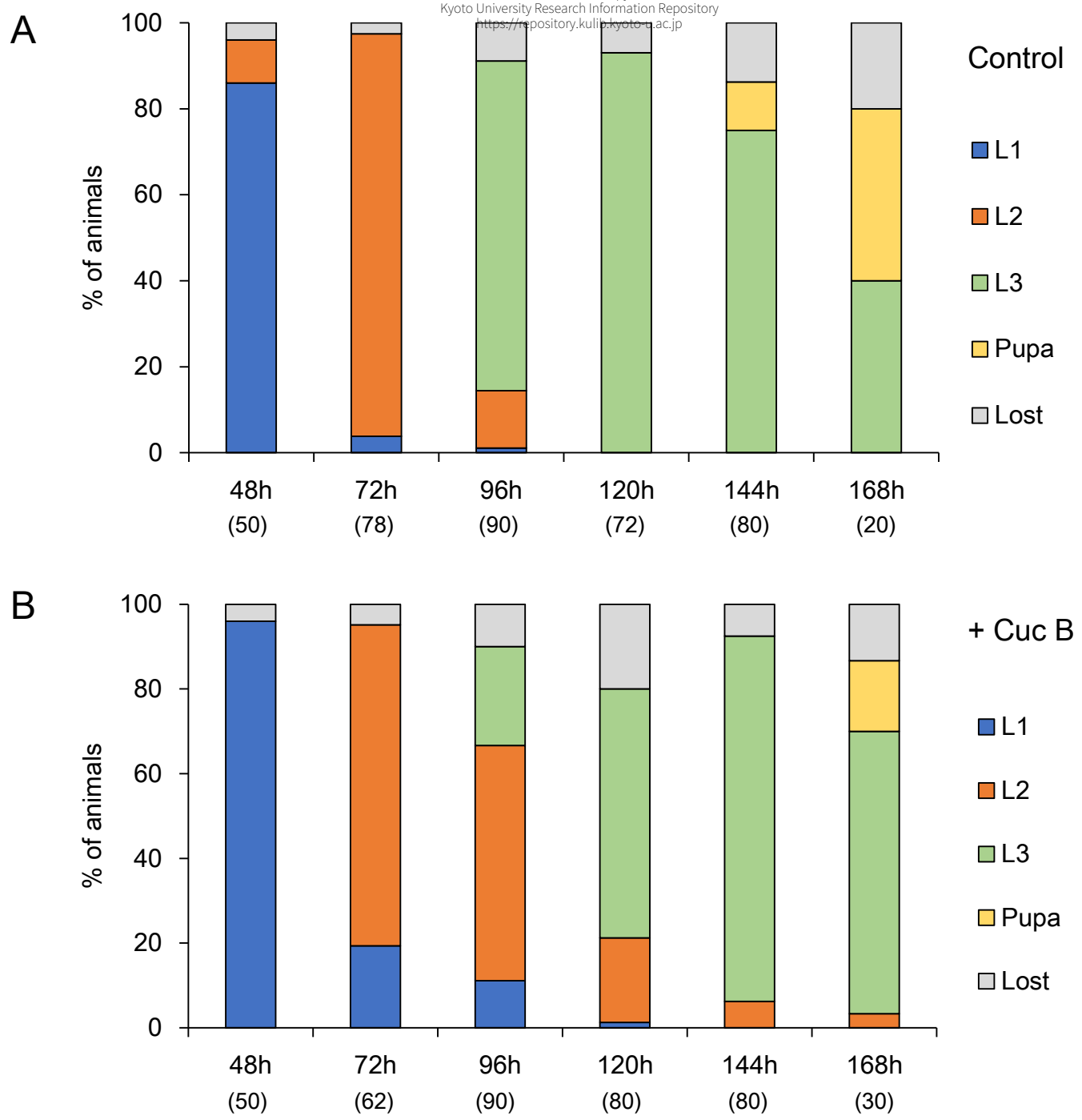

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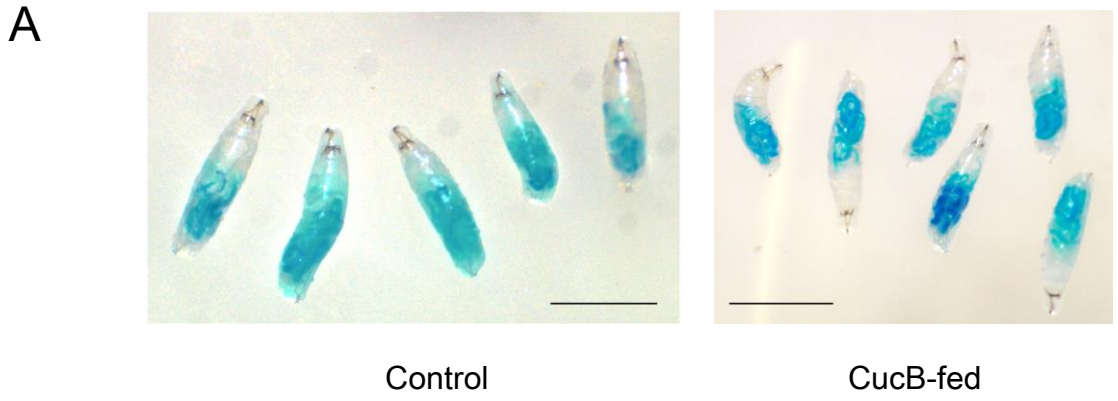

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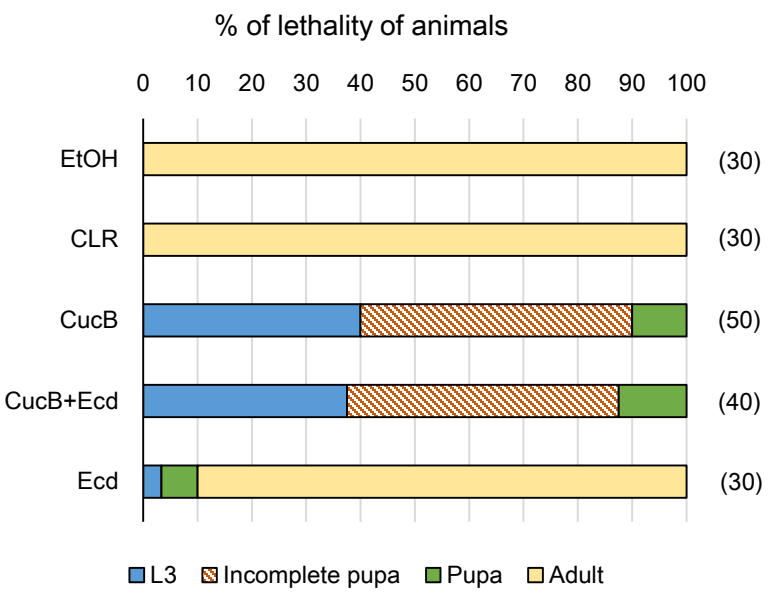
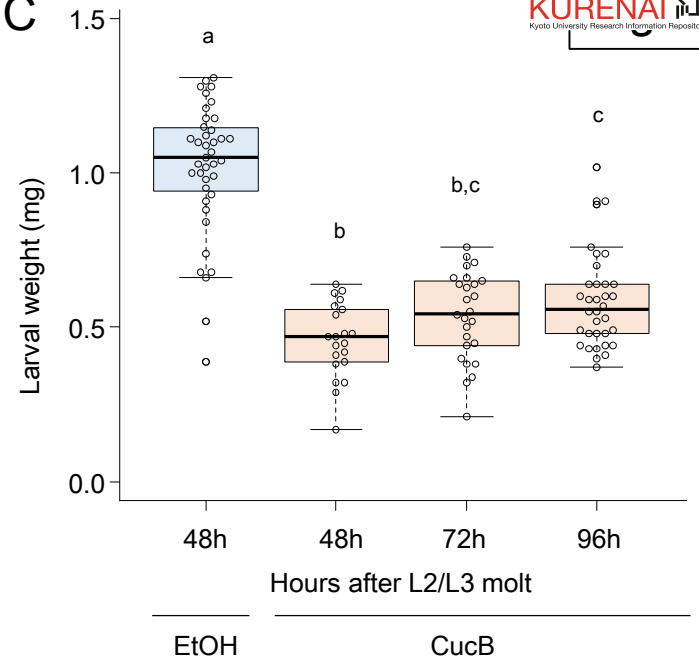
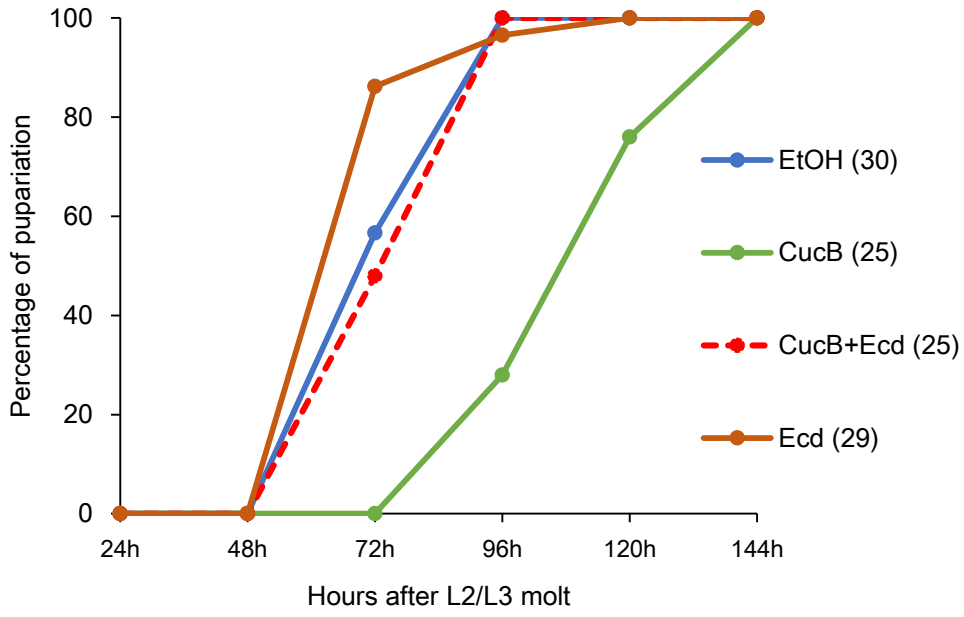


B

C



D



E

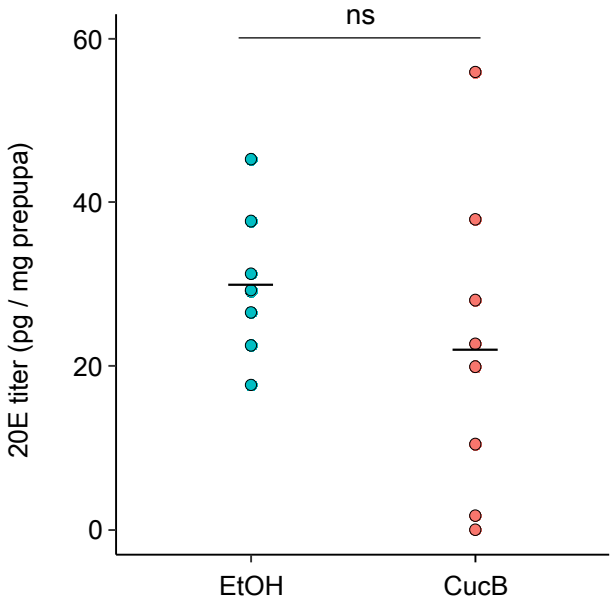


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Fig. 5

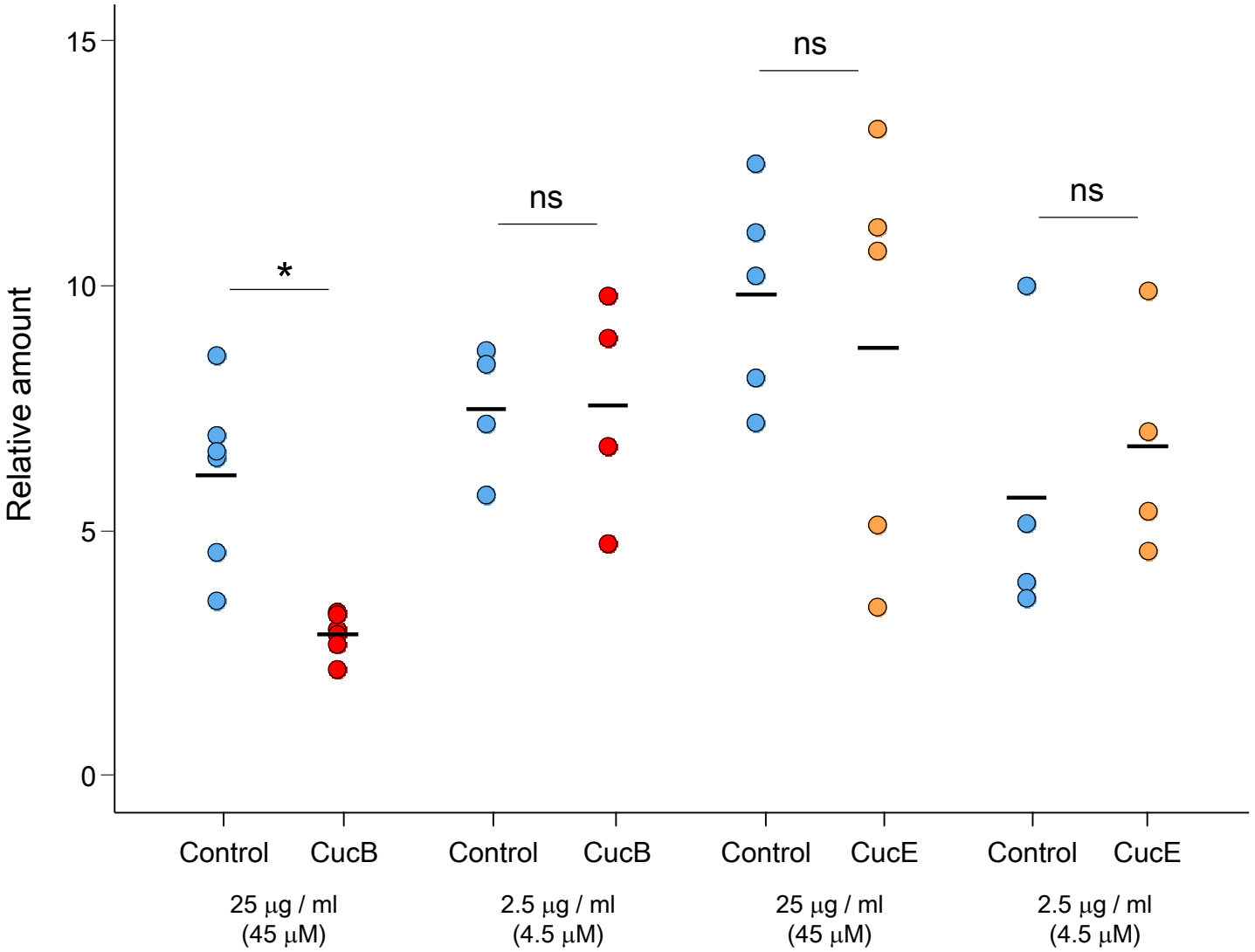

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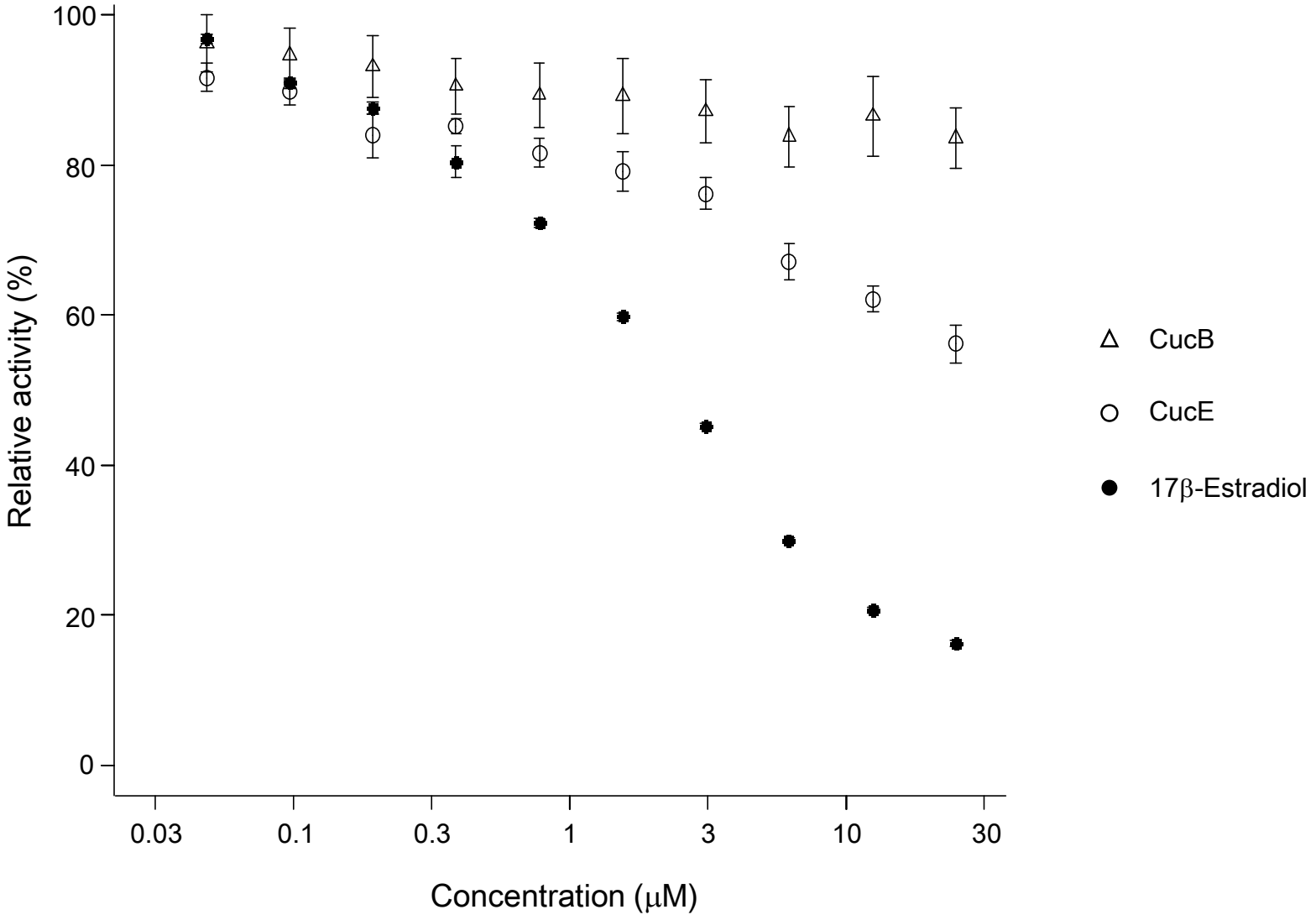
Fig. 6



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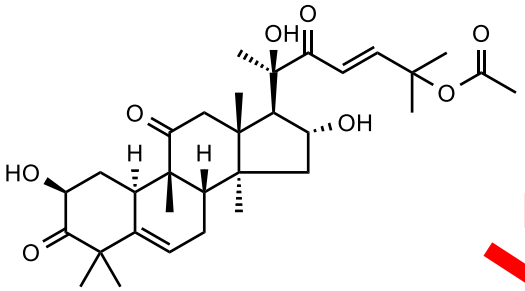
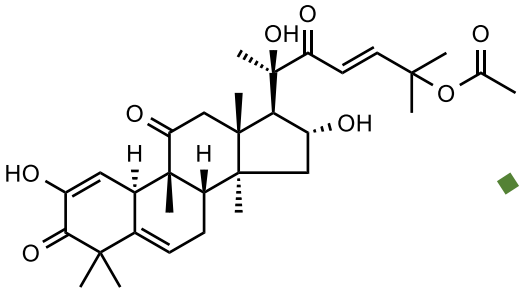
Drosophila melanogaster

Larval development

Inhibition



Cucurbitacin B

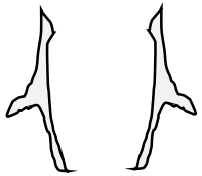


Cucurbitacin E

Inhibition

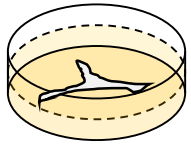
No effect

Bombyx mori



Prothoracic glands

In vitro culture



Ecdysone biosynthesis