Fungal Ecdysteroid-22-oxidase, a New Tool for Manipulating Ecdysteroid Signaling and Insect Development

doi: 10.1074/jbc.M112.341180

Fungal ecdysteroid-22-oxidase: a new tool for manipulating ecdysteroid signaling and insect development

Manabu Kamimura[‡1], Hitoshi Saito[‡2], Ryusuke Niwa[§], Teruyuki Niimi[¶], Kinuko Toyoda[‡3], Chihiro Ueno[‡], Yasushi Kanamori[‡4], Sachiko Shimura[‡], and Makoto Kiuchi[‡]

[‡]From National Institute of Agrobiological Sciences, Owashi, Tsukuba, Ibaraki 305-8634, Japan
[§]Initiative for the Promotion of Young Scientists' Independent Research, Graduate School of Life and Environmental Sciences, University of Tsukuba, Tennoudai, Tsukuba, Ibaraki 305-8572, Japan
[¶]Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa, Nagoya 464-8601, Japan

*Running title: Fungal ecdysteroid inactivation enzyme

[1]To whom correspondence should be addressed: Manabu Kamimura, Division of Insect Science, National Institute of Agrobiological Sciences, 1-2 Owashi, Tsukuba, Ibaraki 305-8634, Japan, Tel.: +81(298) 38-6073; Fax: +81(298) 38-6028; E- mail: kamimura@affrc.go.jp

Keywords: ecdysone, metamorphosis, diapause, embryogenesis, *Bombyx mori*, *Nomuraea rileyi*

Background: Artificial reduction of internal ecdysteroid titer is very difficult to achieve in insects.

Results: Injection of *Nomuraea rileyi* ecdysteroid-22-oxidase (E22O) or forced expression of *E22O* gene reduced ecdysteroid titer and manipulated embryogenesis, molting, metamorphosis, and diapause in a number of insects.

***Conclusion**:* E22O is the first versatile ecdysteroid titer-decreasing tool.

***Significance**:* E220 will be used to answer various ecdysteroid-associated developmental and physiological questions.

SUMMARY

Steroid hormones ecdysteroids regulate varieties of developmental processes in insects. Although the ecdysteroid titer can be increased experimentally with ease, its artificial reduction, though desirable, is very difficult to achieve. Here we characterized the ecdysteroid-inactivating enzyme ecdysteroid-22-oxidase (E22O) from the entomopathogenic fungus *Nomuraea rileyi* and used it to develop methods for reducing ecdysteroid titer and thereby controlling insect development. *Km* and *Kcat* values of the purified E22O for oxidizing ecdysone were 4.4 μM and 8.4/s, respectively, indicating that E22O can inactivate ecdysone more efficiently than other ecdysteroid inactivating enzymes characterized so far. The cloned *E22O* cDNA encoded a FAD-dependent oxidoreductase. Injection of recombinant E22O into the silkworm *Bombyx mori* interfered with larval molting and metamorphosis. In the hemolymph of E22O-injected pupae, the titer of hormonally active 20-hydroxyecdysone decreased and concomitantly large amounts of inactive 22-dehydroecdysteroids accumulated. E22O injection also prevented molting of various other insects. In the larvae of the crambid moth *Haritalodes basipunctalis*, E22O injection induced a diapause-like developmental arrest, which, as in normal diapause, was broken by chilling. Transient expression of the *E22O* gene by *in vivo* lipofection effectively decreased the 20-hydroxyecdysone titer and blocked molting in *B. mori.* Transgenic expression of *E22O* in *Drosophila melanogaster* caused embryonic morphological defects, phenotypes of which were very similar to those of the ecdysteroid synthesis deficient mutants. Thus, as the first available simple but versatile tool for reducing the internal ecdysteroid titer, E22O could find use in controlling a broad range of ecdysteroid-associated developmental and physiological phenomena.

In insects, the steroid hormones ecdysteroids, primarily 20-hydroxyecdysone (20E), play important roles in the regulation of various developmental and physiological processes such as embryogenesis, molting, metamorphosis, reproduction and diapause (1-4). Titers of ecdysteroids are precisely controlled by a combination of synthetic reactions, most of which proceed in the prothoracic gland, whereas they are inactivated in various peripheral organs (5). In the last decade, eight ecdysteroid synthesis enzymes were identified, mainly from the fruit fly *Drosophila melanogaster* and silkworm *Bombyx mori* (6-16). Five of these enzymes were cytochrome P450 encoded by the so-called *Halloween* genes of *D. melanogater* and mutants of which were all embryonic lethal (17). In contrast, the ecdysteroid inactivation process has received relatively less attention. So far, four ecdysteroid inactivation enzymes have been identified in insects (18-23). Mutations in or knockdown of those genes interfered with the insect metamorphoses (21-23), indicating that both synthesis and inactivation of ecdysteroids are essential for the normal development of insects.

Some insect pathogens also use ecdysteroid inactivation enzymes. The best-known example is the baculoviruses, which express the ecdysteroid UDP-glucosyltransferase that inactivates ecdysteroids via sugar conjugation at position C22 (24). Another example is the entomopathogenic fungus *Nomuraea rileyi* that secretes the ecdysteroid-22-oxidase (E22O) that oxidizes the hydroxyl group at C22 to a carbonyl group (25) (Fig. 1). These pathogens inhibit molting of their hosts by inactivating the host ecdysteroid hormones using these enzymes, presumably to maintain a good physiological condition for growth (25,26)

These ecdysteroid inactivation enzymes could potentially serve as powerful tools in ecdysteroid research, as techniques for both increasing and decreasing internal titers of hormones are essential for analyzing their actions *in vivo*. Indeed, increases in the ecdysteroid titer experimentally, by injecting or feeding exogenous ecdysteroid, have revealed various biological functions and modes of action of this hormone in a number of insect species (1-4). A reduction in the ecdysteroid titer using artificial means is, however, very difficult to achieve in most insects, because the prothoracic glands are intricately-shaped organs and are difficult to remove completely by surgical operation. Furthermore, pharmacological agents that could inhibit synthesis or actions of ecdysteroids in a wide spectrum of insects have not been developed yet, and knockdown of genes encoding the ecdysteroid synthesis enzymes or forced expression of genes encoding the ecdysteroid inactivation enzymes is still difficult, except in *D. melanogaster*. Consequently, we do not know exactly what happens when the internal ecdysteroid titer is artificially decreased in most insects. The ecdysteroid inactivating enzymes could potentially solve this longstanding challenge. Particularly, E22O of *N. rileyi* is a good candidate for this purpose, because the *B. mori* larvae that were injected with the *N. rileyi* conditioned culture medium, which contained E22O activity, showed decreased level of ecdysteroid titer (25).

In this study, we characterized E22O and used it for developing methods to reduce the ecdysteroid titer. Injection of recombinant E22O protein or forced expression of *E22O* gene reduced the titer of 20E and blocked embryogenesis, larval molting, larval-pupal or pupal (nymphal)-adult metamorphoses in a number of insects. In addition, injection of E22O induced a diapause-like developmental arrest in the crambid moth *Haritalodes basipunctalis* that was indistinguishable from the normal diapause. To the best of our knowledge, this is the first report indicating that a reduction in the ecdysteroid titer is a sufficient endocrinological stimulus to induce diapauses in insects. Thus, our results suggest that E22O, by reducing the ecdysteroid titer, could serve as a powerful tool for researching a variety of ecdysteroid-dependent phenomena during insect development.

EXPERIMENTAL PROCEDURES

Insects and fungus- The F_1 hybrid strain C145 x N140 of *Bombyx mori* maintained in NIAS and *Lucilia sericata* purchased from a fishing-tackle store were reared on an artificial diet for silkworm (Nihon Nosan Kogyo). *Naxa seriaria* and *Riptortus clavatus* were collected in Tsukuba, Japan and reared on *Ligustrum obtusifolium* leaves and soybeans, respectively. *Tenebrio molitor* was purchased from a pet shop and reared on a powdered bird food. These insects were reared at 25°C under a 12 h light: 12 h dark (12L:12D) photoperiod. *Haritalodes basipunctalis* was collected in Tsukuba and reared on *Firmiana simplex* leaves at 25°C under 16L:8D photoperiod (non-diapausing condition) or at 17°C under 8L:16D photoperiod (diapause-inducing condition). *D. melanogaster* were reared on a standard agar-cornmeal medium at 25°C under 12L:12D photoperiod. Mutant strain *hsp70-GAL4* was obtained from the *Drosophila* Genetic Resource Center at Kyoto Institute of Technology. Mutant strains *engrailed -GAL4* and *Actin5C-GAL4* were obtained from the Bloomington Stock Center. The *Actin5C-GAL4* strain was originally established by Dr. Yasushi Hiromi at the National Institute for Genetics, Japan. *N. rileyi*, maintained in NIAS, was cultured in a medium containing 20 g of maltose, 5 g of tryptone and 5 g of yeast extract in 1 L water. Conditioned media for *N. rileyi* were prepared as described previously (25).

Ecdysteroids and brassinosteroids- Ecdysone (Sigma) and 20-hydroxyecdysone (Mitaka Pharmaceutical Co.) were purified using HPLC (LC-10AT, Shimadzu). Each ecdysteroid was applied to a C_{18} reverse-phase column (TSK gel ODS-80Ts, 4.6 x 150 mm, TOSOH) and then eluted with a 20 to 30% linear gradient of acetonitrile using a flow rate of 0.6 ml/min. Fractions corresponding to each ecdysteroid were pooled, methanol was evaporated off, and dried ecdysteroid was weighed and then dissolved in ethanol. 22-Dehydroecdysone and 22-dehydro-20-hydroxyecdysone were synthesized by reacting ecdysone and 20-hydroxyecdysone, respectively, with the conditioned media of Sf9-E22O cells. They were then purified by HPLC as above, weighed, and dissolved in ethanol. HPLC analysis confirmed that these ecdysteroids were >99% pure.

Ponasterone A (Invitrogen), brassinolide (Wako), and castasterone (Wako) were dissolved in ethanol.

Those ecdysteroid and brassinosteroid solutions were diluted with distilled water and reacted with E22O-containing solutions.

E22O activity assay- An aliquot of E22O-containing solution was mixed with an equal volume of 200 μM of ecdysone and incubated at 25°C. Ten min later, double volume of ethanol was added to stop the reaction and the mixture was centrifuged at 18,000*g* for 10 min. Ecdysone and synthesized 22-dehydroecdysone remaining in the supernatant were separated by HPLC and the amount of 22-dehydroecdysone was calculated from the ratios of peak areas of the two ecdysteroids. *Km* and *Vmax* values were calculated using the GraphPad Prism 5 program (GraphPad Software).

Purification of E22O- The *N. rileyi* conditioned medium (152 ml) was mixed with a protease inhibitor cocktail and solid ammonium sulfate was then added to achieve 50% saturation, and the mixture was continuously stirred at 4°C over night. After centrifugation at 10,000*g* for 20 minutes, E22O was purified from the resulting supernatant by HPLC (Model Bio-HPLC system, TOSOH). The crude extract was applied to a HiTrap Phenyl-Sepharose HP column (Amersham Pharmacia Biotech) equilibrated with buffer A [20 mM Tris-HCl (pH 7.6)] containing 50% ammonium sulfate. The column was eluted using a linear gradient (50 - 0%) of ammonium sulfate in buffer A. Fractions containing E22O were pooled, dialyzed against buffer A containing 0.3M NaCl, and concentrated using an Ultrafree Biomax-5 Centrifugal filter (Millipore). The concentrated solution was then applied to a Superdex 200 pg (1.6 × 60 cm) column equilibrated with buffer A containing 0.3M NaCl. Fractions containing E22O were pooled and concentrated using the Ultrafree Biomax-5 Centrifugal filter again. The concentrated E22O was further purified using a HiTrap Q column (Amersham Pharmacia Biotech). Fractions

containing E22O (0.58 mg) were pooled and used for biochemical characterization. The molecular mass of the purified E22O was estimated by running it on a 15% polyacrylamide gel containing 0.1% SDS along with molecular weight marker proteins as standards. The molecular mass of E22O was also estimated by comparing its retention time on gel-filtration column (Superdex 200 prep grade, 10 mm x 30 cm column, equilibrated with buffer a containing 0.3 M NaCl) with the retention times of the molecular weight marker proteins.

Amino acid sequencing- The N-terminal sequence of the purified E22O was determined using a gas phase protein sequencer (Model LF-3400 DT, Beckman). Phenylthiohydantoin derivatives of individual amino acids were identified by reverse phase HPLC. The purified E22O was subjected to in-gel digestion by V8 protease and the products were separated on SDS-PAGE. The N-terminal sequence of one of the protease-digested products was also determined as above.

cDNA cloning of E22O- Total RNA was extracted from the *N. rileyi* mycelia using TRIzol (Invitrogen) and reverse-transcribed using Ready-To-Go T-Prime First-Strand Beads (Amersham Pharmacia Biotech). A partial *E22O* cDNA was cloned from the cDNA pool by PCR. Forward and reverse PCR primers were designed on the basis of the N-terminal sequences of the purified E22O and its limited V8-proteolysis product, respectively. The first PCR was carried out using the E22O-dF1 (5'-TICCICARGGIGGITGYAG-3') and E22O-dR1 (5'-CAIGCITTITTIACRTTRTG-3') primers. The second nested PCR was carried out using the E22O-dF2 (5'-TGYAGRTGYATICCIGGIGA-3') and E22O-dR2 (5'-TTIACITTITGICCYTGRTC-3') primers. The full-length *E22O* cDNA was obtained by combining 5'-RACE and 3'-RACE reactions with primers that were designed based on the sequences of the partial *E22O* cDNA using SMART RACE cDNA Amplification Kit (Clontech). The nucleotide sequence of the full-length *E22O* cDNA was deposited in the GenBank™/EMBL/DDBJ databases (accession number AB675078). A putative secretion signal of E22O was predicted using the SignalP 3.0 program (27).

Expression of E22O in cultured cell- The entire open reading frame of *E22O* was amplified by RT-PCR from the *N. rileyi* cDNA pool and cloned into the pIZT/V5-His expression vector (Invitrogen), and the resultant plasmid was called pIZT-E22O. The moth *Spodoptera frugiperda* Sf9 cells were transfected with pIZT-E22O using FuGENE HD (Roche Diagnostics). The Sf9 cells transfected with pIZT-E22O were subcultured continuously in IPL-41 medium containing 10% FBS and 300 μg/ml of zeocin. Three months later, all cells acquired resistance against zeocin. We assumed that the plasmid pIZT-E22O got integrated into the genome of the Sf9 cells (designated hereafter as Sf9-E22O cells) and maintained them in IPL-41 medium with 10% FBS and 10 μg/ml of zeocin. When they reached 80-90% confluency, the medium was replaced with IPL-41 without FBS and zeocin. Two weeks later, the conditioned culture medium, which contained a large amount of E22O (Fig. S1*A*) but little FBS and zeocin, was collected. Because the activity of E22O in the medium remained high for a long period at 4 °C (Fig. S1*C*), we stored it in the refrigerator until used later in physiological experiments. Control conditioned medium was similarly prepared from the parental Sf9 cells. In physiological experiments, the conditioned medium of Sf9-E22O or Sf9 cells (5-30 μl) was injected into the hemocoel of insects using a micro-syringe (Hamilton) with a 31 gauge ponit-4 needle.

Transient expression of E22O gene in silkworm- The *E22O* gene was expressed transiently in *B. mori* by *in vivo* lipofection following a slightly modified version of our published procedure (28). Briefly, 2 μg of pIZT-E22O was mixed with 5 μl of Transfast (Promega) in 10 ml of 10 mM Tris-HCl buffer (pH 8.0), incubated for 10 min at room temperature, and then injected into the hemocoel of the silkworm larvae. As the control, the empty pIZT/V5-His plasmid was similarly lipofected into the silkworm larvae.

Transgenic expression of E22O gene in fruit fly-The entire coding region of *E22O* cDNA from pIZT-E22O was cloned into the

Eco RI/*Bgl* II site of the pUAST vector (29). The embryos used as recipients for DNA injection to generate transgenic lines were *yellow white* (*y ac* w^{1118}) flies. Transgenic flies carrying *UAS-E22O* constructs (*UAS-E22O* line) were generated as described previously (30). The *UAS-E22O* line was crossed with different *GAL4* driver lines and F_1 individuals were used for experiments.

Quantification of ecdysteroid titer- Hemolymph samples were individually collected from *B. mori* pupa or *H. basipunctalis* larvae, mixed vigorously with triple volume of methanol, and centrifuged at 18,000*g* for 5 min. *D. melanogaster* embryos were collected as batches 7-12 h after egg laying, a time when embryonic ecdysteroid titer is the highest (31), homogenized in methanol, and then centrifuged. Total ecdysteroid content in the supernatant of these samples were measured by radioimmunoassay using 20E as a standard (32). Supernatant from the *B. mori* sample was run on HPLC and a fraction containing ecdysone, 20E, 22-dehydroecdysone, or 22-dehydro-20-hydroxyecdysone was collected separately. The fractions were dried, dissolved in methanol again, and subjected to radioimmunoassay using each ecdysteroid as a standard. The affinities of the antibody used in radioimmunoassay to ecdysone, 20E, 22-dehydroecdysone, and 22-dehydro-20-hydroxyecdysone were approximately 1: 3: 0.3: 1.

Statistical analysis- Student's t-test was conducted to detect statistically significant differences between E22O treatments and controls using the JMP7 software (SAS Institute).

RESULTS

Purification, kinetic analysis, and cDNA cloning of E22O- As previously shown by us (25), the conditioned culture media of *N. rileyi* exhibited strong E22O activity (Fig. S1*A*). We therefore purified E22O from the *N. rileyi* conditioned medium by HPLC, sequentially using hydrophobic interaction chromatography, gel-filtration chromatography and ion exchange chromatography, and at the end obtained E22O as a single peak (Fig. 2, *A*-*C*). The molecular mass of the purified E22O was estimated to be 76 kDa by both SDS-PAGE and gel-filtration analyses (Fig. 2, *D* and *E*), suggesting that E22O is a monomeric protein.

E22O exhibited highest catalytic activity for ecdysone at pH 10.5, and the activity decreased to one-third of this value at neutral pH (Fig. S2). Because the endogenous E22O normally works at neutral pH in the insect hemolymph, we measured the kinetic properties of the purified E22O at pH 7.0. The *Kcat* of the purified E22O for ecdysone was 8.4/s, which was three times higher than that for 20E; in contrast, the *Km* values were same (i.e., 4.3 μM) for both ecdysteroids (Fig. 2*F*). This observed *Kcat* of the purified E22O for ecdysone was much higher than those of the ecdysteroid UDP-glucosyltransferase of the *Autographa californica* nucleopolyhedrovirus (*Kcat* = 0.069/s) and ecdysone oxidase of the cotton leafworm *Spodoptera littoralis* (*Kcat* = 0.11-0.12/s, calculated assuming that the molecular mass of the enzyme is 190 kDa), while the *Km* values of all three enzymes for ecdysone were similar (19,33). These results suggest that E22O inactivate ecdysone much more efficiently than the two other well-characterized ecdysteroid inactivation enzymes. E22O also oxidized and inactivated other ecdysteroids that contain the hydroxyl group at C22, for example ponasterone A, however, had no effect on plant steroid hormones brassinosteroids, such as brassinolide and castasterone, even though they also have the hydroxyl group at C22 (data not shown).

Next, to clone the *E22O* cDNA, we first sequenced the N-termini of the purified E22O protein and one of the peptides produced by limited hydrolysis of E22O using V8 protease (Fig. S3). Using degenerated primers, designed on the basis of these amino acid sequences, a partial *E22O* cDNA fragment was cloned by RT-PCR. The 5' and 3' ends of the *E22O* cDNA were then cloned using the 5'- and 3'-RACE techniques. The full length *E22O* cDNA encoded a novel FAD-binding oxidoreductase comprising of 594 amino acids (Fig. S3). Consistent with the prediction that E22O is a flavoprotein, purified E22O had a brownish color with an

absorbance at 454 nm. The N-terminal end of E22O contained a putative signal peptide, and the amino acid sequence following it matched with the N-terminal sequence of the purified protein as determined above, suggesting that E22O is processed after the signal peptide and secreted from the cells. The amino acid sequence of E22O was up to 55% identical to those of the alcohol oxidases identified from various fungi (Fig. S3). Although some of these oxidases are involved in the biosynthesis of biologically active agents, such as *Hypomyces subiculosus* alcohol oxidase involved in hypothemycin synthesis and *Fusarium incarnatum* APS9 in apicidin synthesis (34,35), none of them are known to be involved in the modification of steroids or hormones.

Activity of recombinant E22O- When the E22O cDNA was transiently expressed in Sf9 cells, high ecdysone-oxidizing activity was found in the culture media (Fig. S1*A*). Remarkably, 50 μl of the media completely oxidized equal volume of 200 μM (100 μM final concentration) of ecdysone within 10 min (Fig. S1*B*); this final concentration of ecdysone was hundred times higher than the maximal titer found in the *B. mori* pupal hemolymph (Fig. 5*C*). This activity was comparable to or even higher than that observed in the *N. rileyi* conditioned media. In contrast, hardly any activity was observed in the cellular lysates (Fig. S1*A*). These results confirmed that the cloned cDNA encodes E22O and that E22O is a secretory protein.

Next, we generated an Sf9 cell line that stably expressed the *E22O* cDNA (Sf9-E22O cell line) and injected its conditioned medium into *B. mori* individuals to test whether it would decrease the hemolymph ecdysteroid titer and affect their growth. We have previously shown that injection of *N. rileyi* conditioned medium into the mid-penultimate instar larvae of *B. mori* induced precocious pupation (25). Consistent with this observation, some of the larvae that were injected with Sf9-E22O conditioned medium at the beginning of the penultimate instar ate food 3-4 days longer than the control, started spinning during the instar and pupated precociously (Fig. 3*A*). When the conditioned medium was injected into the late-penultimate instar larvae, the last larval ecdysis was inhibited (Fig. 3*B*). Injection of Sf9-E22O conditioned medium into *B. mori* pupae 2 days after pupation prolonged pupal period and interfered with adult emergence (Fig. 3, *C* and *D*). In the hemolymph of the E22O-injected pupae, the 20E titer decreased and instead large amounts of 22-dehydroecdysone and 22-dehydro-20-hydroxyecdysone were present on day 4 when the 20E titer is the highest in the control (Figs. 3*E* and 5*C*). These results indicate that the conditioned media of Sf9-E22O cells can also be used to reduce the 20E titer and thereby manipulate the growth of *B. mori*

Molt inhibition by E22O in various insects- We next examined how E22O would affect the developmental programs in insects other than *B. mori*. E22O-injected last instar larvae of a geometrid moth *Naxa seriaria* (Lepidoptera) remained as larvae for much longer period than the controls, and eventually died without initiating pupation (Fig. 4, *A* and *B*). Similar results were obtained with the last instar larvae of the blowfly *Lucilia sericata* (Diptera) and bean bug *Riptortus clavatus* (Hemiptera). In both species, the larval-pupal or nymphal-adult metamorphosis was rarely observed after E22O injection, while larvae or nymphs injected with the control medium completed metamorphosis within 2 weeks (Fig. 4, *C*-*F*). Injection of E22O into the penultimate or last instar larvae of the yellow mealworm *Tenebrio molitor* (Coleoptera) completely suppressed larval molting and 70% of them died as pharate pupae (Table 1). Furthermore, 70% of E22O-injected prepupae died without completing pupation. E22O injection into *T. molitor* pupae interfered with the normal adult eclosion and many deformed adults with folded or heavily curled wings emerged. Thus, E22O blocked molting and metamorphosis of additional four species belonging to different orders.

Transient expression of E22O gene in the silkworm- We have recently established an *in vivo* lipofection method to express foreign genes in *B. mori* larvae (28). Using this technique, the *E22O* gene was

expressed in *B. mori* 3rd instar larvae. Four days after the lipofection, 0.098 pmol/μl/min (=46 ng/ml/min, N=2) of ecdysone-oxidizing activity was observed in the plasma of the *E22O*-expessing larvae, indicating that *E22O* gene was successfully expressed in larval tissues and expressed E22O protein was secreted into hemolymph. The *E22O*-expressing larvae continued eating in the instar and grew much bigger than the control 3rd instar larvae (Table 2, Fig. S4*A*). Some of them pupated precociously after ecdysis to the 4th instar larvae and feeding food for a week, 3-4 days longer than the control 4th instar larvae (Fig. S4*B*). Similar results were obtained by expressing *E22O* in the 4th instar larvae (Fig. S4*C*).

Next, the *E22O*-expressing plasmid was lipofected into the spinning last instar larvae. Whereas most of these larvae pupated normally, 85% of the pupae could not complete the pupal-adult metamorphosis (Fig. 5*A*). Half of these pupae grew to pharate adults but could not eclose from the pupal case normally (Fig. S4*D*).

We compared the ecdysteroid titers in the hemolymph of the *E22O*-expressing and control pupae. In controls, the total ecdysteroid titer increased after pupation, reached a peak (~ 5 μg/ml) on day 4, and then decreased to the basal level (Fig. 5*B*); the ecdysone and 20E titers on the other hand peaked at around 500 ng/ ml on days 2-3 and on day 4, respectively, and then decreased rapidly (Fig. 5*C*). These temporal changes of total ecdysteroid, ecdysone, and 20E were similar to those observed in *Manduca sexta* pupae (36). The sum of the ecdysone and 20E titers was 800 ng/ ml at the maximum, suggesting that the hemolymph contained much more amounts of other ecdysteroids that could react with the antibody used in RIA.

In contrast, the total ecdysteroid titer continued to increase in the hemolymph of the *E22O*-expressing pupae (Fig. 5*B*). The maximal ecdysone titer (~400 ng/ml) was observed on day 2, as in the controls, but thereafter the titer maintained a level of more than 100 ng/ml (Fig. 5*D*). The 20E titer did not show any obvious peak, but 100-300 ng/ml of 20E was present throughout the pupal period. The maximal 20E titer in the *E22O*-expressing pupae was thus half of that in the controls. While 22-dehydroecdysone and 22-dehydro-20-hydroxyecdysone were not detected in the controls, large amounts of 22-dehydroecdysteroids, particularly 22-dehydroecdysone, accumulated in the hemolymph of the *E22O*-expressing pupae (Fig. 5*D*). The affinity of the antibody used in RIA to 22-dehydroecdysone was one-tenth that to 20E (see EXPERIMENTAL PROCEDURES) and therefore the 20E equivalent values of the 22-dehydroecdysone titers were expected to be very small, suggesting that much amounts of unidentified ecdysteroids were present also in the hemolymph of the *E22O*-expressing pupae. Taken together, these results indicate that transient expression of *E22O* dramatically altered the temporal patterns of the ecdysteroid titers, and affected both larval molting and metamorphosis in *B. mori*. An injection of purified 22-dehydroecdysone or 22-dehydro-20-hydroxyecdysone into late pupae had no effects (data not shown), suggesting that not the accumulation of those 22-dehydroecdysteroids but the reduction of 20E at the peak time or its sustained presence caused the developmental abnormalities.

Transgenic expression of E22O gene in the fruit fly- We also examined the effects of overexpression of *E22O* gene in the fruit fly *Drosophila melanogaster* using the *GAL4/UAS* gene expression system (29). When the *UAS-E22O* line was crossed with the *engrailed-GAL4* line, most (99.8%) of the F_1 individuals were embryonic lethal. They developed to around stage 14 and completed segmentation, but then stopped further differentiation. The denticle belt was not formed in 75% of them (Fig. 6*A*). Similar results were obtained when the *UAS-E22O* line was crossed with the *hsp70-GAL4* line even without heat shock or crossed with the *Actin5C-GAL4* line (data not shown). These phenotypes were very similar to those of the ecdysteroid biosynthesis deficient mutants of *Drosophila* such as *disembodied*, *shadow, phantom, spook* and *shroud* (17), suggesting that the ecdysteroid contents in the *E22O*-expressing embryos were reduced. In fact, at the peak time of the embryonic ecdysteroid titer in wild type (7-12 h after egg laying,

corresponding to stages 12-15) (31), the total ecdysteroid titer in the *engrailed-GAL4>UAS-E22O* embryos was around half that of wild type embryos (Fig. 6*B*).

Induction of diapause in Haritalodes basipunctalis by E22O injection- Although it is widely accepted that low ecdysteroid titer is important for the maintenance of larval and pupal diapause (4), it is still unclear whether a reduction of ecdysteroid titer is sufficient to induce diapause. To resolve this issue, we examined the effects of E22O injection on larval diapause of the crambid moth *H. basipunctalis*.

H. basipunctalis larvae entered diapause in the last larval instar after the wandering behavior when reared at 17 °C under a short day length (diapausing-inducing condition), whereas they pupated and then emerged as adults when reared at 25 °C under a long day length (non-diapausing condition) (Fig. 7, *A* and *F*). First, we verified that the hemolymph ecdysteroid titer is kept low in the diapausing *H. basipunctalis* larvae. In the non-diapausing last instar larvae, the total ecdysteroid was less than 20 ng/ ml during the feeding period (Fig. 7*L*). It increased drastically after the wandering behavior and peaked at around 600 ng/ml one day before pupation. In the diapausing last instar larvae, the ecdysteroid titer was less than 20 ng/ ml during the feeding period as in the non-diapausing larvae (Fig. 7*L*). It increased slightly after the wandering behavior but did not exceed 40 ng/ml for subsequent four weeks. Thus, the hemolymph ecdysteroid titer was kept at a much lower level in the diapausing larvae than in the non-diapausing larvae as expected. Those differences in the ecdysteroid titers between the non-diapausing and diapausing *H. basipunctalis* larvae were very similar to those observed in other insects that enter diapause facultatively, such as larvae of *Pimpla instigator* (37) and *Ostrinia nubilalis* (38,39) and pupae of *M. sexta* (40), *Sarcophaga argyrostoma* (41), *Boettcherisca peregrina* (42), and *Mamestra brassicae* (43).

When the conditioned medium of Sf9-E22O cells was injected into the non-diapausing last instar larvae 1 day after the wandering behavior, pupation was delayed and some individuals remained at the larval stage for very long periods (Fig. 7*B*). When those larvae were transferred to diapause-inducing condition or kept at 5 °C, in both cases most of them lived for longer than 2 months as larvae without eating anything (Fig. 7, *E* and *H*). These developmental characteristics were very similar to those of the diapause-destined larvae (Fig. 7, *C*, *F*. and *I*) but distinct from those of the intact non-diapausing larvae (Fig. 7, *A*, *D*, and *G*). Particularly, all of intact non-diapausing larvae transferred to 5 °C died within 2 months, indicating that E22O injection rapidly imparted high cold hardiness to *H. basipunctalis* larvae.

When the E22O-injected larvae were reared under diapause-breaking condition (5 °C for 6 weeks and then transferred to diapause-inducing condition), they pupated and then emerged as adults, as did diapausing larvae (Fig. 5, *J* and *K*). The E22O-injected non-diapausing larvae were thus in a physiologically similar state to that of the diapausing larvae, which strongly suggest that E22O injection induced diapause or a diapause-like state in the *N. basipunctalis* larvae. As far as we know, these results are the first evidence indicating that a reduction in the ecdysteroid titer is a sufficient endocrinological stimulus to induce diapause in insects.

DISCUSSION

We have characterized the ecdysteroid inactivation enzyme E22O from an entomopathogenic fungus *N. rileyi* and have shown that both injection of the recombinant E22O protein and forced expression of the *E22O* gene reduce the internal ecdysteroid titer and affect the development and physiology of several insect taxa. The E22O-modified phenomena included embryogenesis, larval-larval molt, larval-pupal metamorphosis, pupal- or nymphal-adult metamorphosis, and diapause. Thus, E22O influenced most of the major ecdysteroid-regulated events during insect development.

Injection of recombinant E22O protein or transient expression of the *E22O* gene inhibited molting and metamorphosis in various insect species. In *N. seriaria, L.*

sericata and *R. clavatus*, the E22O-injected last instar larvae or nymphs remained at the stage they were injected for a long period until they died without initiating molt. It is most likely that the injected E22O maintained the titers of ecdysteroids in the hemolymph, particularly that of 20E, below the thresholds to induce metamorphosis in these species. In contrast, more than half of E22O-injected *B. mori* and *T. molitor* larvae eventually initiated molting responses, suggesting that E22O was unable to completely suppress the rise of ecdysteroid titer. In fact, lower but significant level of 20E was found in *B. mori* pupae that were injected with E22O or forced to express the *E22O* gene. These results imply that the effectiveness of E22O for inactivating ecdysteroids varies according to the insect species.

Reduction of ecdysteroid titers either by injection of E22O protein or by expression of *E22O* gene induced precocious metamorphosis *in B. mori.* These results may appear strange because precocious metamorphosis is widely known to be a typical phenotype of the lack of juvenile hormone (1). However, it was also reported that the *B. mori* 4th instar larvae, in which the ecdysteroid synthesis was suppressed by using the imidazole compound KK-42, pupated precociously (44). In addition, there are some reports showing that the ecdysteroid deficiency causes precocious metamorphosis also in *D. melanogaster* (45-47). The precocious pupation in *D. melanogaster,* induced as a result of lowered ecdysteroid titer, was explained as follows: due to the low ecdysteroid titer, the penultimate instar larvae were unable to initiate the last larval-larval molt and kept on feeding, and consequently grew to the point where they surpassed the 'critical weight for precocious pupation', although what eventually initiated the precocious pupation still remains unknown (46). Since the 4th larval period of the E22O-treated *B mori* larvae was also prolonged, a similar mechanism may underlie in the induction of precocious pupation in *B. mori*.

Among the multiple applications of E22O tested here, transgenic insects carrying the *E22O* gene seem to be an ideal system for controlling the ecdysteroid titer at any stage of the development. The *E22O*-expressing transgenic *D. melanogaster* were, however, all embryonic lethal irrespective of the *GAL4* driver lines used. These results were in contrast to those of the *ecdysteroid 26-hydroxylase* (*cyp18A1*)-expressing flies, where the time of death changed from the embryonic stage to the last larval instar stage depending on the *GAL4* driver line used for crossing (21). Thus, contrary to our expectation, the *E22O* gene appears too strong to use in transgenic flies, suggesting that mutations to moderate the enzymatic activity of E22O may be necessary for its transgenic use.

Using E22O, we not only confirmed the importance of ecdysteroids in embryogenesis, larval molting and metamorphoses, but also answered an unsolved question associated with diapause. E22O-injected *H. basipunctalis* last instar larvae remained as larvae for a long period as found with *N. seriaria, L. sericata* and *R. clavatus*. We assumed that this was not a simple developmental arrest, but an artificially induced diapause or a diapause-like state based on the following three reasons. First, the E22O-injected *H. basipunctalis* larvae survived much longer than the above three species without eating anything. Second, they acquired high cold-hardiness, a characteristic often observed in insects in diapause (4). Finally, and more importantly, they resumed development after being exposed to chilling condition, which is the environmental stimulus to break diapause in many insect species (4). These developmental and survival characteristics were indistinguishable from those of the normal diapause-destined larvae. Many researches suggested that larval and pupal diapauses are maintained by the ecdysteroid deficiency (37-43). The hemolymph ecdysteroid titer was kept at a low level also in diapausing *H. basipunctalis* larvae. However, the endocrinological cue to trigger diapause is still not very clear while a low ecdysteroid titer is obviously necessary for insects to enter diapause. Our results strongly suggest that a reduction in the ecdysteroid titer is a key signal to coordinately induce multiple diapause-associated physiological

changes in *N. basipunctalis* including developmental arrest, enhancement of cold hardiness and programming to resume development, although more detailed measurement of ecdysteroid titer and examination of other physiological properties would be necessary to achieve the final goal. It would also be interesting to use E22O to examine whether ecdysteroids play a similar role in inducing larval or pupal diapause in other insects.

In conclusion, the potent ecdysteroid inactivation enzyme E22O could be used as an artificial ecdysteroid titer-reducing tool to manipulate multiple ecdysteroid-regulated phenomena. Particularly, injection of recombinant E22O protein, a relatively simple technique, is potentially applicable to all insects and other arthropods. Judicious applications of methods utilizing E22O could obtain answers to a wide range of ecdysteroid-associated developmental and physiological questions, including uncovering new functions of ecdysteroids.

ACKNOWLEDGMENTS. We thank the Bloomington Stock Center and the *Drosophila* Genetic Resource Center at Kyoto Institute of Technology for providing us the fly strains, Dr. Seiji Tanaka, NIAS, for critically reading the manuscript, and Ms. Yuka Ito, NIAS, for maintaining the cell lines.

REFERENCES

1. Riddiford, L. M. (1985) Hormone action at the cellular level. in *Comprehensive Insect Physiology, Biochemistry, and Pharmacology*. (Kerkut, G. A., and Gilbert, L. I. eds.), pp.37-84, Pergamon Press, Oxford
2. Sonobe, H., and Yamada, R. (2004) *Zool. Sci.* **21**, 503-516
3. Raikhel, A. S., Brown, M. R., and Belles, X. (2005) Hormonal control of reproductive processes. in *Comprehensive molecular insect science* (Gilbert, L. I., Iatrou, K., and Gill, S. S. eds.), pp.433-491, Elsevier, Oxford
4. Denlinger, D. L., Yocum, G. D., and Rinehart, J. P. (2005) Hormonal control of diapause. in *Comprehensive molecular insect science* (Gilbert, L. I., Iatrou, K., and Gill, S. S. eds.), pp.615-650, Elsevier, Oxford
5. Lafont, R., Dauphin-Villemant, C., Warren, J. T., and Rees, H. (2005) Ecdysteroid chemistry and biochemistry. in *Comprehensive molecular insect science* (Gilbert, L. I., Iatrou, K., and Gill, S. S. eds.), pp.125-195, Elsevier, Oxford
6. Warren, J. T., Petryk, A., Marques, G., Jarcho, M., Parvy, J. P., Dauphin-Villemant, C., O'Connor, M. B., and Gilbert, L. I. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11043-11048
7. Petryk, A., Warren, J. T., Marques, G., Jarcho, M. P., Gilbert, L. I., Kahler, J., Parvy, J. P., Li, Y., Dauphin-Villemant, C., and O'Connor, M. B. (2003) *Proc. Natl. Acad. Sci. U. S. A.* **100**, 13773-13778
8. Niwa, R., Matsuda, T., Yoshiyama, T., Namiki, T., Mita, K., Fujimoto, Y., and Kataoka, H. (2004) *J. Biol. Chem.* **279**, 35942-35949
9. Warren, J. T., Petryk, A., Marques, G., Parvy, J. P., Shinoda, T., Itoyama, K., Kobayashi, J., Jarcho, M., Li, Y., O'Connor, M. B., Dauphin-Villemant, C., and Gilbert, L. I. (2004) *Insect Biochem. Mol. Biol.* **34**, 991-1010
10. Namiki, T., Niwa, R., Sakudoh, T., Shirai, K., Takeuchi, H., and Kataoka, H. (2005) *Biochem. Biophys. Res. Commun.* **337**, 367-374
11. Ono, H., Rewitz, K. F., Shinoda, T., Itoyama, K., Petryk, A., Rybczynski, R., Jarcho, M., Warren, J. T., Marques, G., Shimell, M. J., Gilbert, L. I., and O'Connor, M. B. (2006) *Dev. Biol.* **298**, 555-570
12. Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H., and Niwa, R. (2006) *Development* **133**, 2565-2574
13. Maeda, S., Nakashima, A., Yamada, R., Hara, N., Fujimoto, Y., Ito, Y., and Sonobe, H. (2008) *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **149**, 507-516
14. Niwa, R., Namiki, T., Ito, K., Shimada-Niwa, Y., Kiuchi, M., Kawaoka, S., Kayukawa, T.,

Banno, Y., Fujimoto, Y., Shigenobu, S., Kobayashi, S., Shimada, T., Katsuma, S., and Shinoda, T. (2010) *Development* **137**, 1991-1999
15. Yoshiyama-Yanagawa, T., Enya, S., Shimada-Niwa, Y., Yaguchi, S., Haramoto, Y., Matsuya, T., Shiomi, K., Sasakura, Y., Takahashi, S., Asashima, M., Kataoka, H., and Niwa, R. (2011) *J. Biol. Chem.* **286**, 25756-25762
16. Ou, Q., Magico, A., and King-Jones, K. (2011) *PLoS Biol.* **9**, e1001160
17. Chavez, V. M., Marques, G., Delbecque, J. P., Kobayashi, K., Hollingsworth, M., Burr, J., Natzle, J. E., and O'Connor, M. B. (2000) *Development* **127**, 4115-4126
18. Takeuchi, H., Chen, J. H., O'Reilly, D. R., Rees, H. H., and Turner, P. C. (2000) *Biochem. J.* **349**, 239-245
19. Takeuchi, H., Chen, J. H., O'Reilly, D. R., Turner, P. C., and Rees, H. H. (2001) *J. Biol. Chem.* **276**, 26819-26828
20. Sonobe, H., Ohira, T., Ieki, K., Maeda, S., Ito, Y., Ajimura, M., Mita, K., Matsumoto, H., and Wilder, M. N. (2006) *J. Biol. Chem.* **281**, 29513-29524
21. Guittard, E., Blais, C., Maria, A., Parvy, J. P., Pasricha, S., Lumb, C., Lafont, R., Daborn, P. J., and Dauphin-Villemant, C. (2010) *Dev. Biol.* **349**, 35-45
22. Rewitz, K. F., Yamanaka, N., and O'Connor, M. B. (2010) *Dev. Cell* **19**, 895-902
23. Yang, H. J., Wang, M. X., Zhang, P., Sabhat, A., Malik, F. A., Bhaskar, R., Zhou, F., Li, X. H., Hu, J. B., Sun, C. G., Niu, Y. S., and Miao, Y. G. (2011) *Arch. Insect Biochem. Physiol.* **78**, 17-29.
24. O'Reilly, D. R. (1995) *Insect Biochem. Mol. Biol.* **25**, 541-550
25. Kiuchi, M., Yasui, H., Hayasaka, S., and Kamimura, M. (2003) *Arch. Insect Biochem. Physiol.* **52**, 35-44
26. Cory, J. S., Wilson, K. R., Hails, R. S., and O'Reilly, D. R. (2001) Host manipulation by insect pathogens: the effect of the baculovirus egt gene on host-virus interaction. in *Endocrine interactions of insect parasites and pathogens* (Edwards, J. P., and Weaver, R. J. eds.), pp.233-244, BIOS Scientific Publishers, Oxford
27. Bendtsen, J. D., Nielsen, H., von Heijne, G., and Brunak, S. (2004) *J. Mol. Biol.* **340**, 783-795
28. Kanamori, Y., Hayakawa, Y., Matsumoto, H., Yasukochi, Y., Shimura, S., Nakahara, Y., Kiuchi, M., and Kamimura, M. (2010) *J. Biol. Chem.* **285**, 36933-36944
29. Brand, A. H., and Perrimon, N. (1993) *Development* **118**, 401-415
30. Rubin, G. M., and Spradling, A. C. (1982) *Science* **218**, 348-353
31. Sullivan, A. A., and Thummel, C. S. (2003) *Mol. Endocrinol.* **17**, 2125-2137
32. Takeda, S., Kiuchi, M., and Ueda, S. (1986) *Bull. Seric. Exp. Stn.* **30**, 361-374 (in Japanese with English summary)
33. Evans, O. P., and O'Reilly D, R. (1998) *Biochem. J.* **330 (Pt 3)**, 1265-1270
34. Reeves, C. D., Hu, Z., Reid, R., and Kealey, J. T. (2008) *Appl. Environ. Microbiol.* **74**, 5121-5129
35. Jin, J. M., Lee, S., Lee, J., Baek, S. R., Kim, J. C., Yun, S. H., Park, S. Y., Kang, S., and Lee, Y. W. (2010) *Mol. Microbiol.* **76**, 456-466
36. Warren, J. T., and Gilbert, L. I. (1986) *Insect Biochem.* **16**, 65-82
37. Claret, J., Porcheron, P., and Dray, F. (1978) *C. R. Acad. Sci. Sci. D* **286**, 639-641
38. Bean, D. W., and Beck, S. D. (1983) *J. Insect Physiol.* **29**, 687-693
39. Gelman, D. B., and Woods, C. W. (1983) *Com. Biochem. Physiol. Part A* **76**, 367-375
40. Bowen, M. F., Bollenbacher, W. E., and Gilbert, L. I. (1984) *J. Exp. Biol.* **108**, 9-24
41. Richard, D. S., Warrent, J. T., Saunders, D. S., and Gilbert, L. I. (1987) *J. Insect Physiol.* **33**, 115-122
42. Moribayashi, A., Kurahashi, H., and Ohtaki, T. (1988) *Com. Biochem. Physiol. Part A* **91**, 157-164
43. Endo, K., Fujimoto, Y., Kondo, M., Yamanaka, A., Watanabe, M., Weihua, K., and Kumagai, K. (1997) *Zool. Sci.* **14**, 127-133
44. Kadono-Okuda, K., Kuwano, E., Eto, M., and Yamashita, O. (1987) *Dev. Growth Differ.* **29**, 527-533
45. Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W. A., and Thummel, C. S. (2002) *Dev. Cell* **3**, 209-220

46. Zhou, X., Zhou, B., Truman, J. W., and Riddiford, L. M. (2004) *J. Exp. Biol.* **207**, 1151-1161
47. Gibbens, Y. Y., Warren, J. T., Gilbert, L. I., and O'Connor, M. B. (2011) *Development* **138**, 2693-2703

Footnotes

[*] This work was supported by a Grant-in-Aid (Bio Design Program) from the Ministry of Agriculture, Forestry and Fisheries, Grants-in-aid for Scientific Research 21580070 and 22380039, and the Special Coordination Funds for Promotion Science and Technology from the Ministry of Education, Culture, Sports, Science, and Technology of Japanese government.
The nucleotide sequence reported in this paper has been submitted to the DDBJ/GenBankTM/EBI Data Bank with accession number AB675078.
This article contains supplemental figures 1-4.
[1]To whom correspondence should be addressed: Manabu Kamimura, Division of Insect Science, National Institute of Agrobiological Sciences, 1-2 Owashi, Tsukuba, Ibaraki 305-8634, Japan, Tel.: +81(298) 38-6073; Fax: +81(298) 38-6028; E- mail: kamimura@affrc.go.jp
[2]Present address: Department of Applied Biology, Kyoto Institute of Technology, Sakyo-ku, Kyoto 606-8585, Japan.
[3]Present address: ZIEBEN CHEMICALS Co., Ltd., Inashiki, Ibaraki 300-0422, Japan.
[4]Present address: *Drosophila* Genetic Resource Center, Kyoto Institute of Technology, Ukyo-ku, Kyoto 616-8354, Japan.
The abbreviations used are: E22O, ecdysteroid-22-oxidase; 20E, 20-hydroxyecdysone

Figure legends:

FIGURE 1. **Enzymatic reactions of E22O.** E22O oxidizes the hydroxyl group at position C22 of ecdysone and 20-hydroxyecdysone to a carbonyl group. The oxidized ecdysteroids lose hormonal activity.

FIGURE 2. **Purification and biochemical characterization of E22O.** *A-C,* E22O was purified from the *N. rileyi* conditioned medium by sequential use of Phenyl-Sepharose HP hydrophobic interaction chromatography (*A*), Superdex 200 pg gel-filtration chromatography (*B*), and HiTrap Q column chromatography (*C*). Fractions indicated using the hatched bars in (*A*) and (*B*) were pooled and purified further in (*B*) and (*C*), respectively. Fractions indicated using the closed bar in (*C*) contained pure E22O; they were pooled and then used for further biochemical analyses. *D, E,* Molecular mass of E22O was determined by SDS-PAGE (*D*) and Superdex-200 pg gel-filtration chromatography (*E*). *F,* Kinetic analysis. Various concentrations of ecdysone or 20-hydroxyecdysone were incubated with 2.5 ng/ 50 μl of purified E22O in phosphate buffer (pH 7.0) and amounts of 22-dehydroecdysteroids formed were measured after 10 min. *Kcat* values were calculated using the molecular mass of E22O as 76 kDa.

FIGURE 3. **Effects of E22O injection on growth and hemolymph ecdysteroid titer of *B. mori*.** *A,* Precocious pupation induced by injection of 30 μl of Sf9-E22O conditioned medium into newly molted penultimate (4th) instar larvae. Left: a control pupa metamorphosed from an intact 5th instar larva; right: a miniature precocious pupa induced by E22O injection. Ten % of the E22O-injected larvae started spinning a week later and half of them pupated precociously. *B,* Molting of the penultimate instar larvae after injecting 30 μl of the conditioned medium of either Sf9 (control) or Sf9-E22O cells just before the head capsule slippage (day 3.5). *C,* Pupal development after injecting 30 μl of the conditioned medium of either Sf9 (control) or Sf9-E22O 2 days after pupation. *D,* Pupal period of control and E22O injected-pupae. Only pupae that eclosed as adults normally were included in the calculation. Asterisks (**) indicate that the values are significantly different ($p<0.01$) by Student's t-test. *E,* Ecdysteroid titers of control and E22O injected-pupae on day 4 (2 days after the medium injection). Error bars represent SEM (N=4).

FIGURE 4. **Effects of E22O injection on the growth of three insects.** Last instar larvae or nymphs were injected with 30 μl (for *N. seriaria*) or 5 μl (for *L. sericata* and *R. clavatus*) of conditioned culture medium of either the control Sf9 cell cells (*A, C, E*) or Sf9-E22O cells (*B, D, F*), and subsequently their growth was observed. Eighty to 160 individuals were used for each experiment.

FIGURE 5. **Effects of transient *E22O* expression in spinning silkworm on the growth and ecdysteroid titer.** *A,* Effects of E22O expression on the pupal development. Plasmid pIZT-E22O and control plasmid pIZT/V5-his were introduced separately by *in vivo* lipofection into the spinning last instar larvae and developments of normally pupated individuals were observed. *B,* Changes in the total ecdysteroid titer (20E equivalent) in the hemolymph of non-lipofected control and E22O-expressing pupae. *C, D,* Changes in the titer of each ecdysteroid in the hemolymph of the non-lipofected control (*C*) and *E22O*-expressing pupae (*D*). Error bars represent SEM (N=3-8).

FIGURE 6. **Effects of transgenic *E22O* expression in *D. melanogaster* embryo.** *A,* A typical phenotype of *engrailed-GAL4>UAS-E22O* embryo. Left: wild type, right: *engrailed-GAL4>UAS-E22O*. Note that even though the segmentation was completed, the denticle belt was not formed in the transgenic fly. *B,* Comparison of the total ecdysteroid titer (20E equivalent) between the wild type and *engrailed-GAL4>UAS-E22O* embryos. The ecdysteroid titer was measured in batches of eggs collected 7-12 hours after the oviposition. Error bars represent SEM (N=6-8). Asterisks (**) indicate that the values are significantly different ($p<0.01$) by Student's t-test.

FIGURE 7. **Growth, survival, and ecdysteroid titer of *H. basipunctalis* larvae under different conditions.** Non-diapausing larvae reared under non-diapausing conditions (*A, D, G*), non-diapausing larvae injected with 30 μl of the conditioned culture medium of the Sf9-E22O cells 1 day after

wandering (*B, E, H*), and diapause-destined larvae reared under diapause-inducing conditions (*C, F, I*) were reared under non-diapausing condition (*A-C*), diapause-inducing condition (*D-F*), or at 5°C (*G-I*) after wandering, and their subsequent growth and survival were monitored. The E22O-injected non-diapausing larvae (*J*) and intact diapause-destined larvae (*K*) were reared also under diapause-breaking condition and their growth was monitored. Sixty to 300 larvae were used for each experiment. *L*, Changes in the total ecdysteroid titer (20E equivalent) in the hemolymph of non-diapausing and diapausing last instar larvae. Larvae were reared under the non-diapausing and diapause-inducing conditions continuously. Their growth are shown in A and F, respectively. Error bars represent SEM (N=2-17).

Table 1. Effects of injection of Sf9-E22O conditioned medium into *Tenebrio molitor*.

Injected stage	Injected medium	N	Total number of larval molts	%							
				Dead larva[2)]	Dead prepupa	Unsuccessful pupation	Dead pupa	Dead pharate adult	Unsuccessful eclosion	Deformed adult[3)]	Normal adult
Larvae[1)]	Control medium	84	45	33	0	0	2	0	0	0	64
	E22O medium	83	0	27	43	28	0	0	0	0	2
Prepupae	Control medium	111	-	-	3	8	2	1	0	0	86
	E22O medium	79	-	-	49	24	4	8	1	14	0
Pupae	Control medium	43	-	-	-	-	2	0	5	0	93
	E22O medium	79	-	-	-	-	13	10	0	73	4

[1)]This includes both penultimate and last instar larvae.

[2)]Individuals that died within 3 days of the injection were exclued from data.

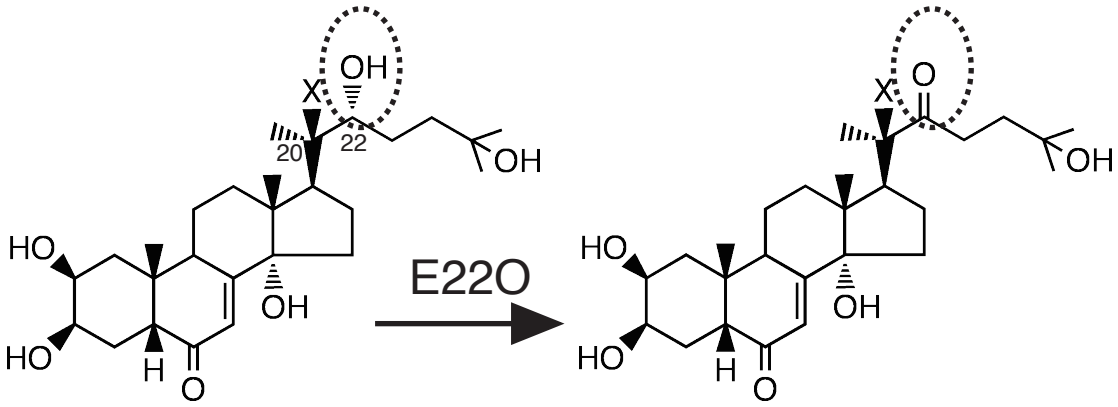
[3)]This category includes adults with folded or heavily curled wings.

Table 2. Effects of *E22O* gene expression on the growth of *B. mori* larvae. The *E22O* cDNA was introduced into *B. mori* by *in vivo* lipofection at the beginning of 3rd larval instar, and larval growth was subsequently monitored.

			%							
			Death during 3rd instar				Death during 4th instar			
Plasmid	N	Average 3rd instar period (day)1)	Larval death (1-5 days after injection)	Larval death (6-10 days after injection)	Larval death (>10 days after injection)	Unsuccessful ecdysis	Larval death	Unsuccessful ecdysis	Precocious spinning	Ecdysis into last instar
Control plasmid	110	4.7	1	0	0	0	5	1	0	93
pIZT-E22O	110	7.1	2	6	19	6	25	2	7	34

[1)]Only larvae that molted normally into the 4th instar were included in the calculation. The two values are significantly different ($p<0.01$) by Student's t-test.

Fig. 1



X= H: Ecdysone
X= OH: 20-Hydroxyecdysone

Fig. 2

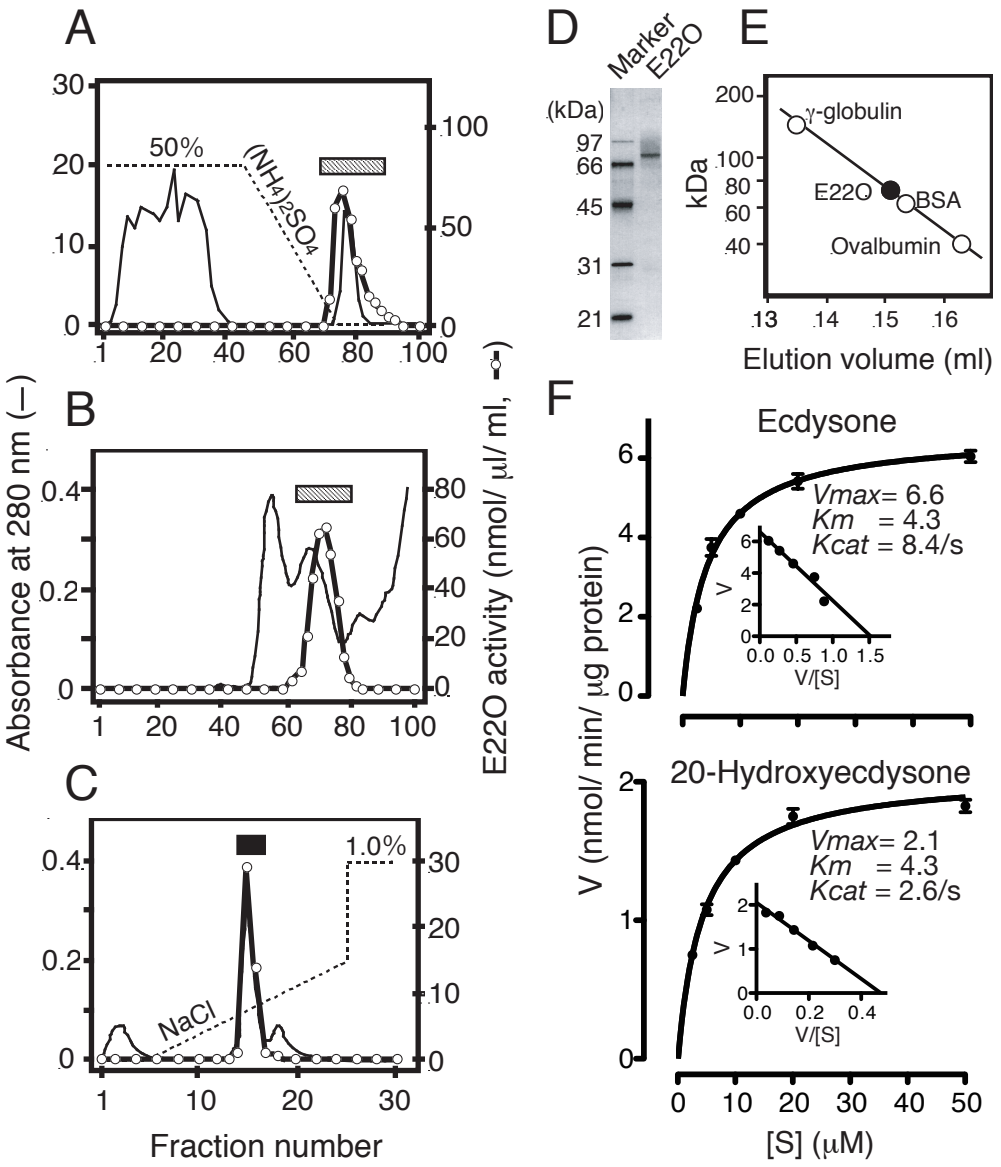

A
B
C
D
E
F
50%
(NH4)2SO4
1.0%
NaCl
Absorbance at 280 nm (–)
E22O activity (nmol/ μl/ ml, -■-)
Fraction number
Marker
E22O
(kDa)
97
66
45
31
21
kDa
γ-globulin
BSA
Ovalbumin
Elution volume (ml)
Ecdysone
Vmax= 6.6
Km = 4.3
Kcat = 8.4/s
20-Hydroxyecdysone
Vmax= 2.1
Km = 4.3
Kcat = 2.6/s
V (nmol/ min/ μg protein)
V/[S]
[S] (μM)

Fig. 3

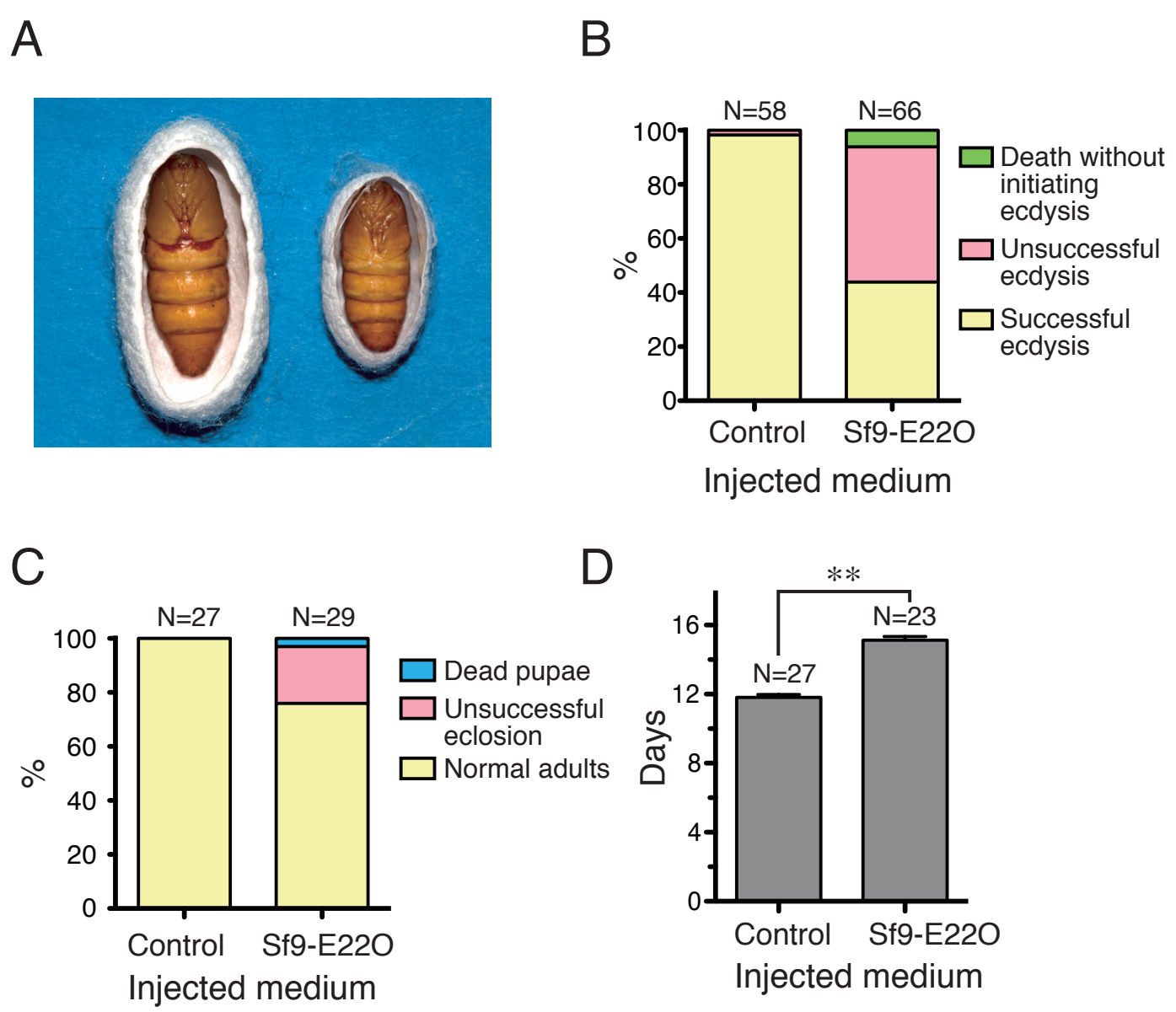

A
B
N=58
N=66
100
80
60
40
20
0
%
Death without initiating ecdysis
Unsuccessful ecdysis
Successful ecdysis
Control
Sf9-E22O
Injected medium
C
N=27
N=29
Dead pupae
Unsuccessful eclosion
Normal adults
Control
Sf9-E22O
Injected medium
D
**
N=27
N=23
16
12
8
4
0
Days
Control
Sf9-E22O
Injected medium
E
6000
4000
2000
0
1200
800
400
0
ng/ ml
Control
E22O-injected
Total ecdysteroid
Ecdysone
20-Hydroxyecdysone
22-Dehydroecdysone
22-Dehydro-20-hydroxyecdysone
Ecdysteroid

Fig. 4

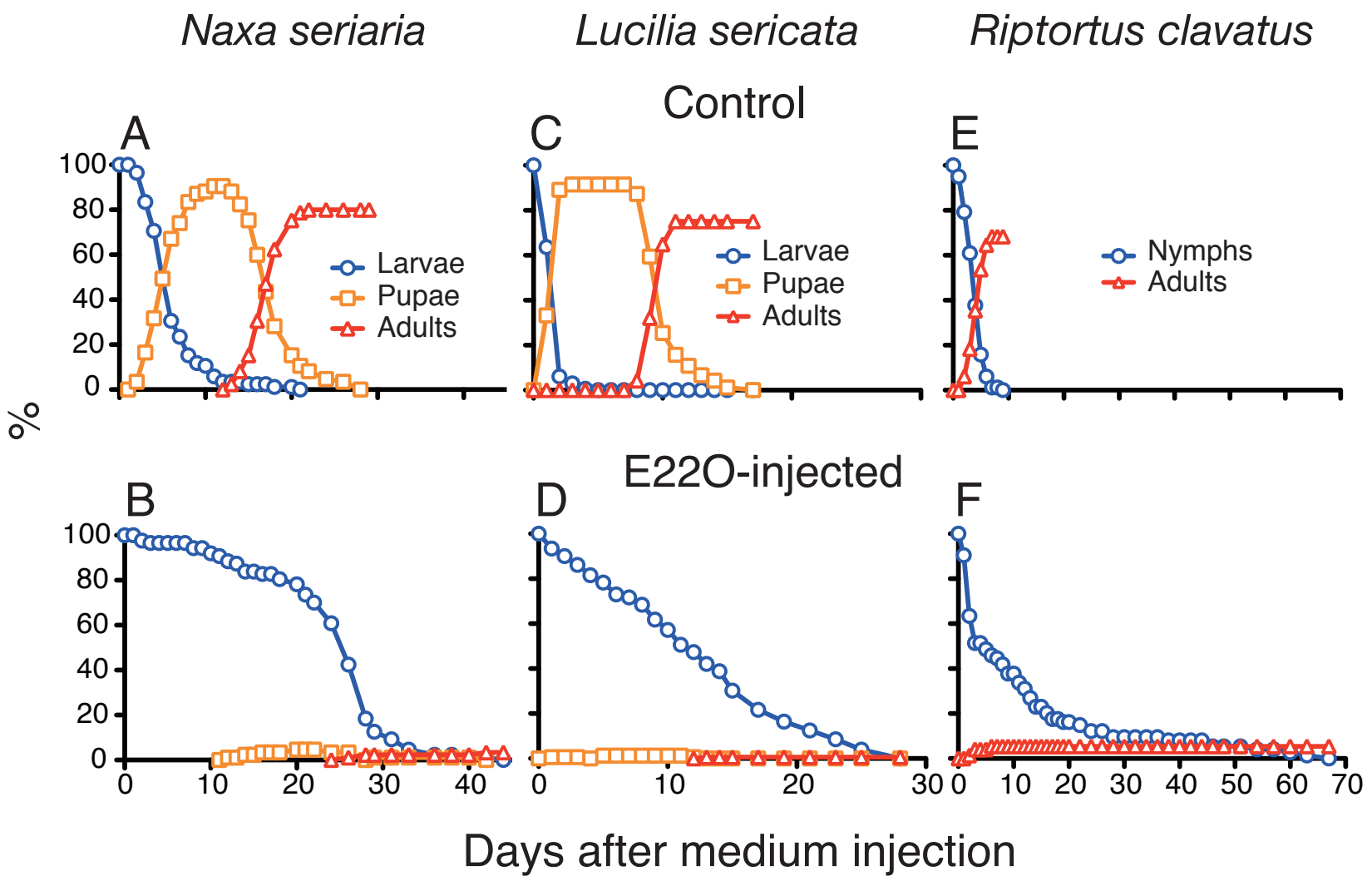

Naxa seriaria
Lucilia sericata
Riptortus clavatus
Control
E22O-injected
A
B
C
D
E
F
%
100
80
60
40
20
0
Larvae
Pupae
Adults
Nymphs
0 10 20 30 40
0 10 20 30
0 10 20 30 40 50 60 70
Days after medium injection

Fig. 5

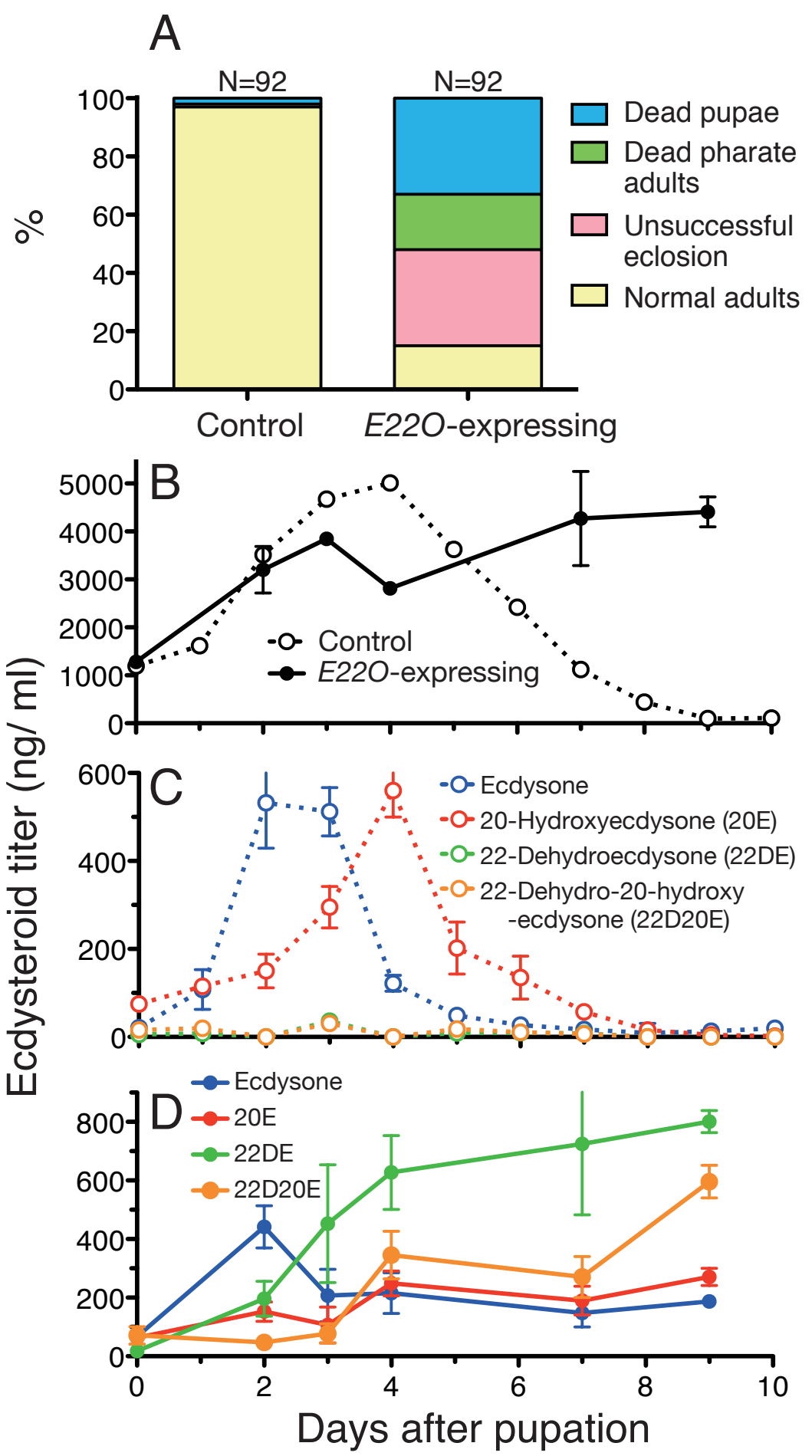
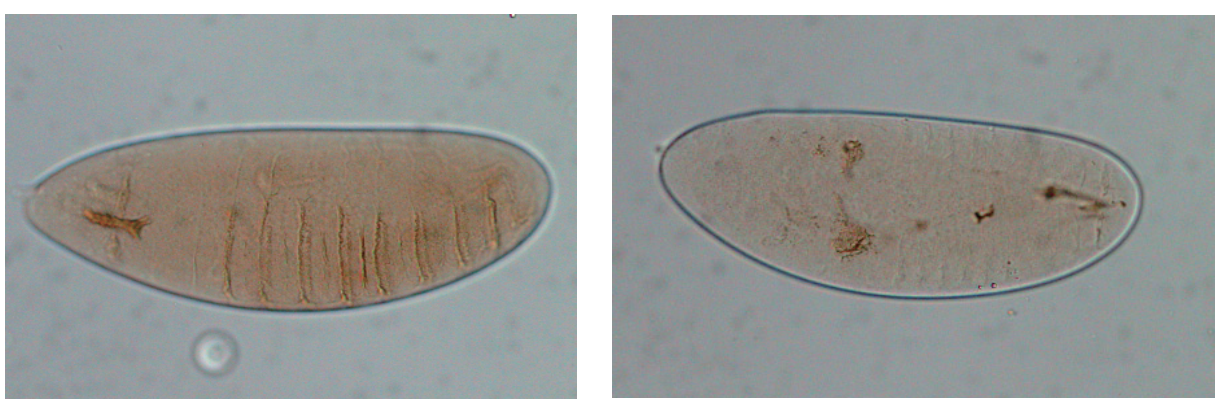

A
N=92
N=92
100
80
60
40
20
0
%
Dead pupae
Dead pharate adults
Unsuccessful eclosion
Normal adults
Control
E22O-expressing
B
5000
4000
3000
2000
1000
0
Control
E22O-expressing
Ecdysteroid titer (ng/ ml)
C
600
400
200
0
Ecdysone
20-Hydroxyecdysone (20E)
22-Dehydroecdysone (22DE)
22-Dehydro-20-hydroxy -ecdysone (22D20E)
D
800
600
400
200
0
Ecdysone
20E
22DE
22D20E
0
2
4
6
8
10
Days after pupation

Fig. 6

A


Wild type
engrailed-GAL4>UAS-E22O

B

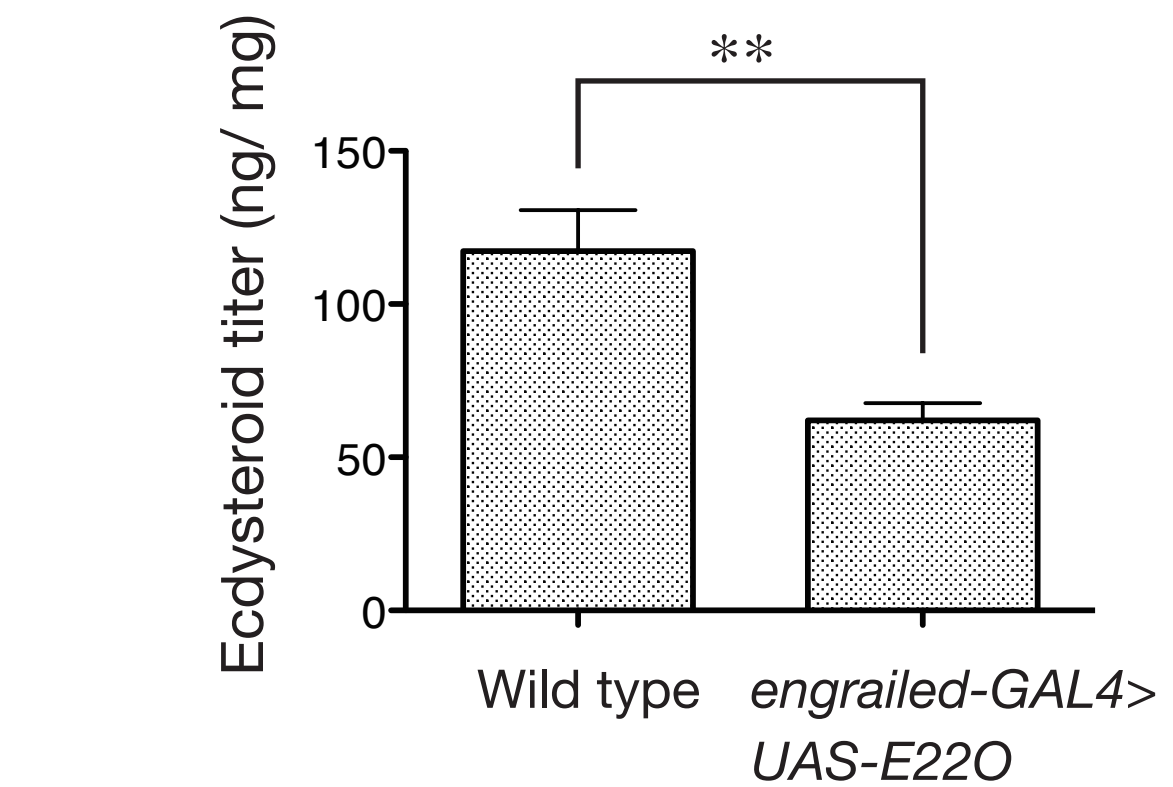
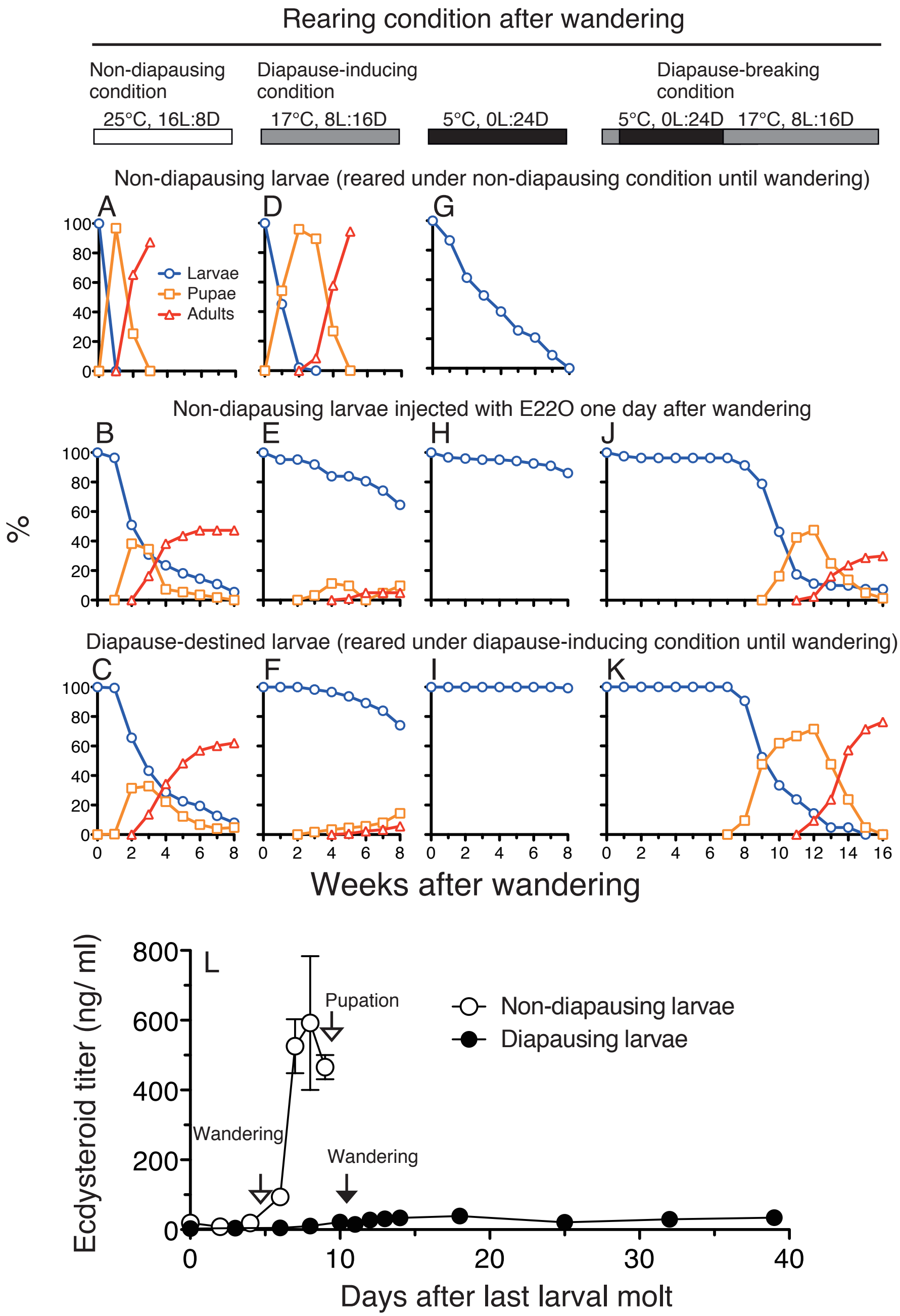

**
Ecdysteroid titer (ng/ mg)
150
100
50
0
Wild type
engrailed-GAL4>
UAS-E22O

Fig. 7


Rearing condition after wandering
Non-diapausing condition
25°C, 16L:8D
Diapause-inducing condition
17°C, 8L:16D
5°C, 0L:24D
Diapause-breaking condition
5°C, 0L:24D
17°C, 8L:16D
Non-diapausing larvae (reared under non-diapausing condition until wandering)
A
D
G
Larvae
Pupae
Adults
Non-diapausing larvae injected with E22O one day after wandering
B
E
H
J
Diapause-destined larvae (reared under diapause-inducing condition until wandering)
C
F
I
K
%
Weeks after wandering
L
Pupation
Wandering
Wandering
Non-diapausing larvae
Diapausing larvae
Ecdysteroid titer (ng/ ml)
Days after last larval molt