Developmental Biology 429 (2017) 71–80

Contents lists available at ScienceDirect

Developmental Biology

journal homepage: www.elsevier.com/locate/developmentalbiology

RNAi-mediated knockdown of *SPOOK* reduces ecdysteroid titers and causes precocious metamorphosis in the desert locust *Schistocerca gregaria*

Ryohei Sugahara*, Seiji Tanaka, Takahiro Shiotsuki

National Agriculture and Food Research Organization, Institute of Agrobiological Sciences, 1-2 Ohwashi, Tsukuba, Ibaraki 305-8634, Japan

ARTICLE INFO

Keywords:
Desert locust
Halloween gene
Precocious metamorphosis
Ecdysteroid
Juvenile hormone
Short wings

ABSTRACT

The *Halloween* gene *SPOOK* (*SPO*) is involved in the production of the active metabolite of ecdysteroid, 20-hydroxyecdysone (20E), in insects. A previous study showed that RNAi-mediated knockdown of *SPO* in *Schistocerca gregaria* last instar nymphs markedly reduced the hemolymph 20E titer, but did not affect metamorphosis. In the present study, the effects of *SPO* interference on development were re-examined in this locust. Injections of *SPO* double-stranded RNA (*dsSPO*) into nymphs at mid and late instars significantly delayed nymphal development and interfered with molting. The 20E levels of *dsSPO*-treated nymphs were generally low, with a delayed, small peak, suggesting that disturbance of the 20E levels caused the above developmental abnormalities. A small proportion of the *dsSPO*-injected nymphs metamorphosed precociously, producing adults and adultoids. Precocious adults were characterized by small body size, short wings with abbreviated venation, and normal reproductive activity. Fourth instar nymphs that precociously metamorphosed at the following instar exhibited temporal expression patterns of *ecdysone-induced protein 93F* and the juvenile hormone (JH) early-inducible gene *Krüppel homolog 1* similar to those observed at the last instar in normal nymphs. Adultoids displayed mating behavior and adultoid females developed eggs, but never laid eggs. JH injection around the expected time of the 20E peak in the *dsSPO*-injected nymphs completely inhibited the appearance of adultoids, suggesting that appearance of adultoids might be due to a reduced titer of JH rather than of 20E. These results suggest that *SPO* plays an important role in controlling morphogenesis, metamorphosis, and reproduction in *S. gregaria*.

1. Introduction

Insect molting and metamorphosis are regulated by two major hormones, ecdysteroid and juvenile hormone (JH) (Truman and Riddiford, 2002). Generally, an increase in the ecdysteroid level in the absence of JH causes larvae and nymphs to undergo metamorphosis. In contrast, in the presence of JH, which inhibits metamorphosis, it induces larval-larval and nymphal-nymphal molts. The active metabolite of ecdysteroid, 20-hydroxyecdysone (20E), transmits the molting signal to the nuclear receptor complex consisting of the ligand-binding ecdysone receptor (EcR) and its heterodimeric partner ultraspiracle (Nakagawa and Henrich, 2009). Thus, regulation of the 20E titer plays a key role in determining the timings of molts in insects.

20E is synthesized from cholesterol and/or plant sterols by cytochrome P450 enzymes encoded by the *Halloween* genes such as *SPOOK* (*SPO*), *PHANTOM* (*PHM*), *SHADE* (*SHD*), *SHADOW* (*SAD*), and *DISEMBODIED* (*DIB*) (Niwa and Niwa, 2014). Thus, the *Halloween* genes play essential roles in insect development. Knockdown of the *Halloween* genes delays or completely prevents nymphal or larval molts in the fruit fly *Drosophila melanogaster*, the commercial silkmoth *Bombyx mori*, and the small brown planthopper *Laodelphax striatellus* (Enya et al., 2014; Jia et al., 2013; Niwa et al., 2010; Yoshiyama et al., 2006). In the desert locust *Schistocerca gregaria,* RNAi-mediated knockdown of *SPO*, *PHM*, and *SHD* markedly reduced the hemolymph 20E titer in the last nymphal instar, but did not affect metamorphosis, and knockdown nymphs successfully ecdysed to the adult stage without showing any abnormality (Marchal et al., 2012, 2011). These findings have led researchers to hypothesize that the 20E peak preceding a molt is dispensable for molt induction (De Loof et al., 2012). On the other hand, EcR is necessary for successful metamorphosis in this locust (Lenaerts et al., 2016).

We have been interested in the molecular mechanism controlling phase polyphenism in locusts (Sugahara et al., 2016, 2015b; Tanaka et al., 2016). In preliminary studies, we have examined the potential role of 20E in the regulation of certain genes that are likely involved in body-color polyphenism, and we have attempted to lower the 20E titer

* Corresponding author.
E-mail addresses: rsugahara@affrc.go.jp, fancryo@yahoo.co.jp (R. Sugahara).

http://dx.doi.org/10.1016/j.ydbio.2017.07.007
Received 9 March 2017; Received in revised form 20 June 2017; Accepted 16 July 2017
Available online 19 July 2017

by knocking down *SPO* in *S. gregaria* third instar nymphs by using double-stranded (ds)RNA (unpublished observations). Unexpectedly, knockdown of this gene interfered with nymphal development and metamorphosis. Therefore, in the current study, we re-examined the role of *SPO* in *S. gregaria*. We provide evidence that *SPO* plays an important role in controlling morphogenesis, metamorphosis, and reproduction in *S. gregaria*. Interestingly, RNAi-mediated knockdown of *SPO* during the nymphal stage produces precocious adults with short wings. Here, we describe the details of these results and discuss the underlying mechanisms.

2. Materials and methods

2.1. Insects

A Nigerian strain of *S. gregaria* was established from individuals collected in Niger by Prof. H.J. Ferenz in 2004 (Tanaka and Nishide, 2013). An Ethiopian strain of *S. gregaria* was obtained from the Desert Locust Control Organization for Eastern Africa in Addis Ababa, Ethiopia (Tanaka and Yagi, 1997). Locusts were maintained in an air-conditioned room at 31 °C under a 12-h-light/12-h-darkness cycle as described previously (Sugahara et al., 2016). The locusts were fed leaves of rescue grass (*Bromus catharticus*), orchard grass (*Dactylis glomerata*), sorghum (*Sorghum bicolor*), and Japanese mustard spinach (*Brassica rapa* subsp. *perviridis*), depending on the season. Wheat bran and cabbage (*Brassica oleracea*) were supplied continuously. All experiments in this study were carried out with crowd-reared (gregarious) locusts.

2.2. RNAi

A partial sequence of *SPO* was amplified from cDNA synthesized from RNA extracted from the whole body of *S. gregaria* third instar nymphs using the primers listed in Table S1, and was cloned into the pLit vector (Sugahara et al., 2014). The cDNA fragment with T7 promoters at both termini was amplified by PCR and subjected to bi-directional transcription (Sugahara et al., 2015b). dsRNA was synthesized *in vitro* using the T7 RiboMAX large-scale RNA production kit (Promega), according to the manufacturer's protocol. A dsRNA fragment corresponding to the green fluorescent protein Venus (*dsVENUS*) was used as a negative control.

For knockdown of *SPO* in *S. gregaria*, first instar nymphs were injected with 0.5 μL dsRNA solution (1 μg μL^{-1}) twice at days 1 and 3. Larger nymphs were injected with 2 μL dsRNA solution (1 μg μL^{-1}) at day 0 or days 0 and 2. *dsVENUS* was injected into other individuals as controls. Male and female individuals were haphazardly included in each treatment group.

2.3. Observations of wing venation

To observe the fore- and hindwings of normal and precocious adults, the wings were removed from adults, held between two acrylic plates, and photographed with a scanner. The images were observed on a monitor screen by referring to the locust wing venation described by V.M. Dirsh (cited by Uvarov, 1966).

2.4. Quantitative reverse transcription (qRT)-PCR analysis

The mRNA expression levels of the ecdysone-induced protein 93F (E93) and Krüppel homolog 1 (Kr-h1) were examined in fourth instar *S. gregaria* nymphs injected with *SPO* dsRNA (*dsSPO*) at the third instar and non-treated fourth and fifth instar nymphs. *E93* has been identified as an adult specifier gene as the German cockroach, *Blattella germanica*, undergoes supernumerary nymphal ecdysis after knockdown of *E93* (Belles and Santos, 2014; Ureña et al., 2014). Methoprene tolerant protein (Met), a JH receptor, transduces the JH signal by inducing *Kr-h1* expression, and thus the hemolymph JH titer reflects the level of *Kr-h1* expression in *B. germanica* (Lozano and Belles, 2011). Accordingly, these proteins represent metamorphosis regulators and show characteristic expression patterns before metamorphosis (Belles and Santos, 2014; Ureña et al., 2016). Total RNA was extracted from the thorax, *i.e.*, pronotum, mesonotum, and metanotum, of nymphs using ISOGEN (Nippon Gene) and the SV Total RNA Isolation System (Promega). To examine the efficiency of RNAi for *SPO*, RNA was extracted from the prothoracic glands, where *SPO* is highly expressed (Marchal et al., 2011). First-strand cDNA was synthesized from total RNA using SuperScript III reverse transcriptase and a mixture of oligo(dT) primers and random hexamers (all Invitrogen), according to the manufacturer's instructions.

For qRT-PCR analysis, partial sequences of *SPO*, *Kr-h1*, *E93*, and the glyceraldehyde 3-phosphate dehydrogenase gene *GAPDH* were amplified using the primers listed in Table S1, and cloned into the pCR-Blunt II-TOPO or pLit vector. Serial dilutions of pCR-*SPO*, pCR-*Kr-h1*, pLit-*E93*, and pCR-*GAPDH* plasmids were used as standards. The expression of *SPO*, *Kr-h1*, *E93*, and *GAPDH* was quantified using SYBR Premix Ex Taq II kit (Takara Bio) in a LightCycler 480 (Roche Diagnostics KK), as previously described (Sugahara et al., 2015a). The molar amounts of target gene transcripts were calculated based on crossing point analysis, using the standard curves obtained from the above plasmid standards. *SPO*, *Kr-h1*, and *E93* expression levels were normalized against *GAPDH* transcripts.

2.5. Ecdysteroid titer measurements

Hemolymph samples were collected from locusts with a polypropylene tip upon leg amputation. Hemolymph taken from 3 to 9 nymphs of each developmental stage was pooled with a melanization inhibitor, 1-phenyl-2-thiourea (PTU; Wako), in a polypropylene microtube. Three pooled samples were collected for each stage and were stored at −80 °C until use. The hemolymph ecdysteroid concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using a previously described method (Shiotsuki et al., 2005), with minor modifications. The hemolymph samples were mixed with thirty times their volume of methanol and ecdysteroids were extracted after centrifugation at 18,000× *g* for 20 min. Phosphate-buffered saline containing 5% skim milk was used as a blocking reagent. A horseradish peroxidase conjugate of 20E and antiserum against 20E were the same as previously reported (Shiotsuki et al., 2005). A standard curve was generated using 20E (Sigma), and the ecdysteroid concentrations are expressed as 20E equivalents.

2.6. JH titer measurements

Hemolymph samples were collected with a glass capillary tube upon leg amputation. Hemolymph taken from 3 to 9 nymphs of each stage was pooled in a glass vial containing PTU. One pooled sample for days 0 and 1, and two pooled samples for days 2, 3, 4, 5, and 6 of the fourth instar hemolymph were used. All samples were stored at −80 °C until further processing. JH concentrations were measured by liquid chromatography-mass spectrometry (LC-MS) as previously described (Furuta et al., 2013).

2.7. Chemicals

JHIII (Fluka Fine Chemical, Tokyo, Japan) was mixed with peanut oil (100 μg in 2 μL) and injected between the second and third abdominal sternites with a microsyringe.

2.8. Statistical analysis

Differences in the timings of molts and death were evaluated by the Brunner-Munzel test, using the lawstat package included in R (version

3.3.2, R Development Core team 2016). The Mann-Whitney *U*-test in Prism (GraphPad Software Inc., version 5.01) was used to compare egg lengths and gene expression levels. Statistical significance was considered at a P-value < 0.05 (two-sided) for all tests.

2.9. Accession codes

The nucleotide sequences of genes used in the present study are available in the DNA Data Bank of Japan (DDBJ) or GenBank under the following accession numbers: *E93*, LC215000; *Kr-h1*, LC275909; *SPOOK*, JF747520.

3. Results

3.1. RNAi of SPOOK delays molting

To analyze the effects *SPOOK* knockdown on molting, *dsSPO* was injected into *S. gregaria* nymphs at different instars and their development was observed. The RNAi efficiency was confirmed by qRT-PCR; *dsSPO* injection into nymphs significantly reduced *SPO* transcript levels in their prothoracic glands (Fig. S1). Nymphs injected with *dsSPO* at the first instar showed minor delays in the second and third ecdyses post-injection as compared to control (*dsVENUS*-injected) nymphs, although the time to attain the adult stage did not significantly differ between the two groups (Fig. 1A). Nymphs injected with *dsSPO* at the second instar consistently showed a delay in ecdyses to the following instars, including the adult stage, as compared to the controls (Fig. 1B). A conspicuous delay in ecdysis was observed after injections of *dsSPO* at the third instar (Fig. 1C), and some individuals remained at the same (third) instar for > 30 days before they died. Unexpectedly, some of the *dsSPO*-injected nymphs (29%) metamorphosed precociously to the fifth instar as described in detail below. Approximately half of the *dsSPO*-injected nymphs went through five nymphal instars before emerging as adults, as was observed in the controls. Injections of *dsSPO* into fourth instar nymphs caused a marked developmental delay (Fig. 1D). In this case, 30% of individuals became nymph-adult intermediates or adultoids after the following ecdysis and underwent no further molting. Details of the morphological and reproductive characteristics of these individuals are described further in this section. A portion (33%) of the nymphs injected with *dsSPO* at the fourth instar attained the adult stage after molting twice, as observed in the controls. The locusts injected with *dsSPO* at the fifth (last nymphal) instar also showed a marked delay in ecdysis to the following (adult) stage as compared to the controls (Fig. 1E); however, they looked normal otherwise.

3.2. Hemolymph titer of 20E in dsSPO-injected nymphs

To determine the effect of *SPO* knockdown on the 20E titer, the hemolymph 20E titer was measured after injection of *dsSPO* or *dsVENUS* at day 0 of the fourth instar. Most of the *dsVENUS*-injected nymphs molted to the fifth instar five days later. Their hemolymph 20E titer during the fourth instar started to increase at day 2 and peaked at day 3 (Fig. 2). In contrast, 20E titers in the *dsSPO*-injected nymphs were generally low during the first four days, with a small peak at day 5; two days later than in the controls (day 3). These results suggested that the delayed molting observed in *dsSPO*-injected nymphs compared to controls was correlated with a delay in peak titer of 20E in the hemolymph.

3.3. Injection of dsSPO at the third instar induces precocious metamorphosis

Injection of third instar nymphs with *dsSPO* brought about three types of developmental fate: *i.e.*, 1) normal-looking adults (41%), 2) precocious adults (30%), and 3) permanent nymphs (27%) (Fig. 3A). In the first type, locusts emerged as normal-looking adults after going through five nymphal instars, as observed for the *dsVENUS*-injected controls. The second type comprised locusts that emerged as adults precociously after the fourth nymphal instar. They were characterized by a small body size and short wings (Fig. 3B). Locusts of the last type were permanent nymphs that eventually died without molting. One of the permanent nymphs survived for as long as 50 days post injection.

Injection of third instar nymphs with *dsSPO* resulted in curled wing pads at the fourth instar in 28% of the nymphs that attained this stage (Fig. 3C). Additionally, they differed from control (normal) nymphs in the sizes and shapes of the white markings on the pronotum (Fig. 3D); the white markings in normal individuals were bow-shaped, whereas those in nymphs with curled wing pads were barrel-shaped. The nymphs with curled wing pads metamorphosed precociously.

Normal fifth instar nymphs have five eye stripes; precocious adults at the fifth instar were characterized by five eye stripes, whereas normal adults, which corresponded to the sixth instar, had six eye stripes (Fig. 3E). The precocious and normal-looking adults were reared separately to observe changes in body color and behavior related to reproduction. At two weeks after the last molt, the body color of precocious adult males started changing from light brown to yellow (n = 9), a symptom of sexual maturation typically observed in crowd-reared normal adults (Pener, 1991), and they copulated with precocious adult females (n = 10; Fig. 3F). The mated females laid eggs that were significantly smaller than those laid by control females (Fig. 3G). Although only few hatchlings emerged from these eggs, most of them were green, whereas all hatchlings emerging from the eggs of control females were black (Fig. 3H).

3.4. Expression patterns of metamorphosis-associated genes

To determine whether *SPO* knockdown affected the expression patterns of other genes associated with metamorphosis, the transcript levels of *E93* and *Kr-h1* were measured in nymphs injected or not with *dsSPO* at the third instar. *E93* expression levels in non-treated (control) nymphs were relatively low during the fourth instar and increased during the fifth (last) instar (Fig. 3I). In nymphs injected with *dsSPO* at day 0 of the third instar, *E93* levels during the fourth instar were significantly higher in locusts with curled wing pads but lower in those with normal wing pads than in non-treated individuals (Mann-Whitney *U*-test, $P < 0.05$ in both tests). The level of *Kr-h1* expression in non-treated nymphs was relatively high in the fourth instar and declined in the fifth instar (Fig. 3J), a pattern similar to that observed in the last two nymphal instars in *B. germanica* (Belles and Santos, 2014; Lozano and Belles, 2011; Ureña et al., 2016). In *dsSPO*-injected locust nymphs, *Kr-h1* expression in the fourth instar was lower in the nymphs with curled wing pads than in the non-treated nymphs at the same instar (Mann-Whitney *U*-test, $P < 0.05$). In contrast, the levels in *dsSPO*-injected nymphs with normal wing pads were similar to those in the non-treated nymphs (Mann-Whitney *U*-test, $P > 0.05$). These results suggest that *dsSPO*-injected fourth instar nymphs with normal wing pads and those with curled wing pads are physiologically similar to non-treated nymphs at the penultimate (fourth) and the last (fifth) instar, respectively.

3.5. Characteristics of precocious adult wings

Although the forewings of precocious adults were much smaller than those of normal adults, the main veins were phenotypically similar (Fig. 4A). In contrast, the compartments between the anterior and posterior medias of the precocious forewing were reduced in number and size as compared to those of the normal forewing. For example, the area between the anterior and posterior medias of the normal forewing contained double rows of compartments with several columns, whereas the corresponding area in the precocious forewing contained a single row, with fewer columns (Fig. 4A). Similar differences between

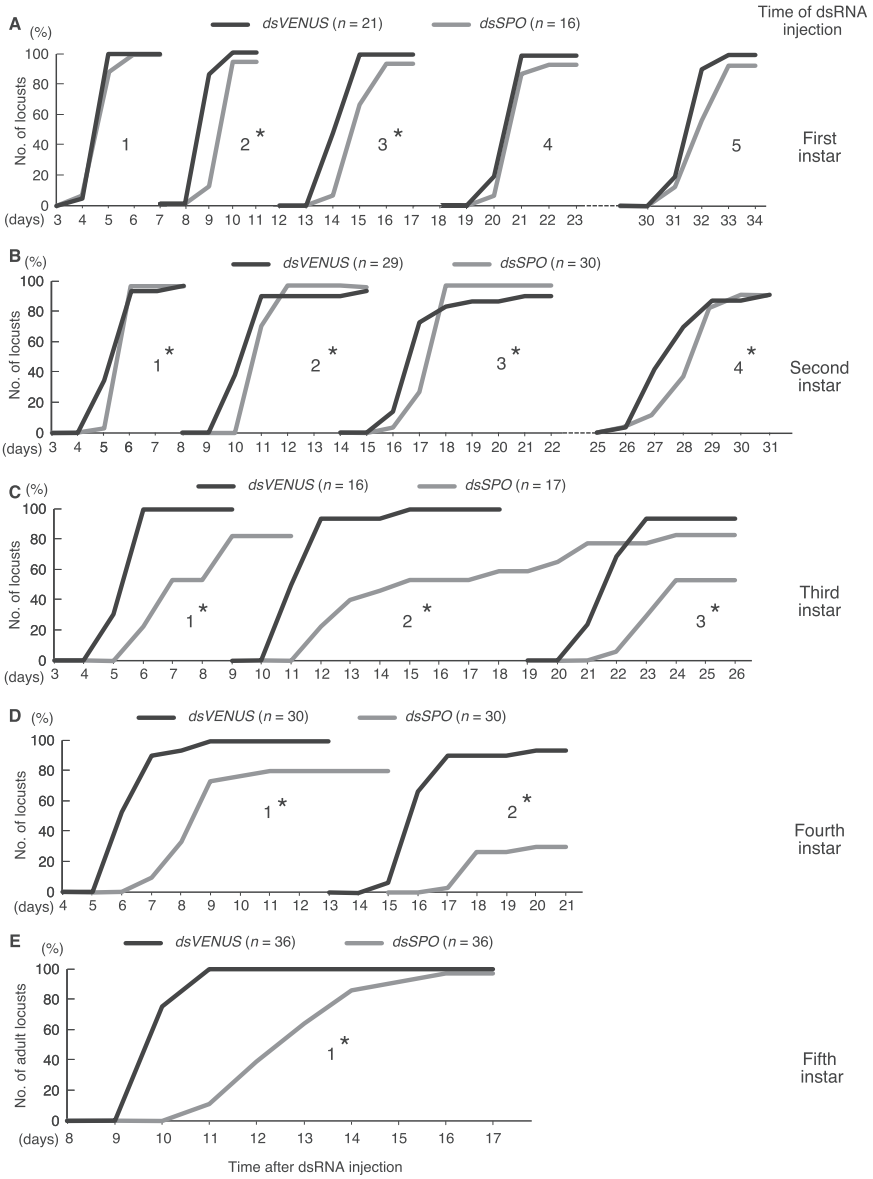

Fig. 1. Effects of *dsSPO* injections into nymphs at different instars on the times of *S. gregaria* molts. Nymphs at the first (A), second (B), third (C), fourth (D), and fifth instar (E) were injected with *dsSPO* or *dsVENUS* twice, and the times of molts were recorded every day. The numbers in each panel indicate the number of molts after injections. The horizontal axis indicates the time in days after the first injection. The numbers of test locusts are shown above the graphs. Significant differences in the time of molting between *dsVENUS*- and *dsSPO*-injected nymphs were evaluated with the Brunner–Munzel test (*$P < 0.05$).

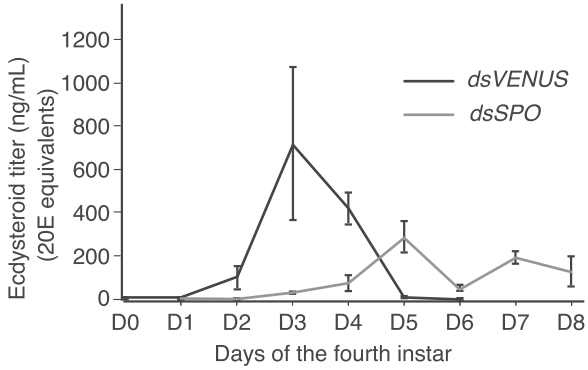

Fig. 2. Effects of *SPO* knockdown on the ecdysteroid titer in *S. gregaria*. Temporal fluctuations in hemolymph 20E concentration in fourth instar nymphs injected with *dsSPO* or *dsVENUS* at day 0 of the fourth instar as determined by ELISA. Error bars represent the standard errors (SEs; $n = 3$ each).

precocious and normal forewings were observed in other areas (not shown). Moreover, the hindwings of precocious adults were smaller than those of normal adults (Fig. 4B). As observed for the forewings, the main veins were present in the hindwings of both precocious and normal adults, but some of the compartments were apparently abbreviated in the precocious hindwing.

Fig. 4C and D illustrate the relative lengths of forewings in normal-looking and precocious adults that were injected with *dsSPO* at the third instar. In this study, the E/C value (E, the length of the forewing; C, maximum head width) was used to compare the relative wing lengths of these adults, after Tanaka and Nishide (2012). The frequency distribution of E/C values for *dsSPO*-injected females showed a bimodal pattern (Fig. 4C). Precocious adults displayed smaller E/C values, while normal-looking adults had larger E/C values. Similar results were obtained for males (Fig. 4D).

3.6. Injection of dsSPO at the fourth instar induces adultoids

A substantial number of individuals injected with *dsSPO* at the

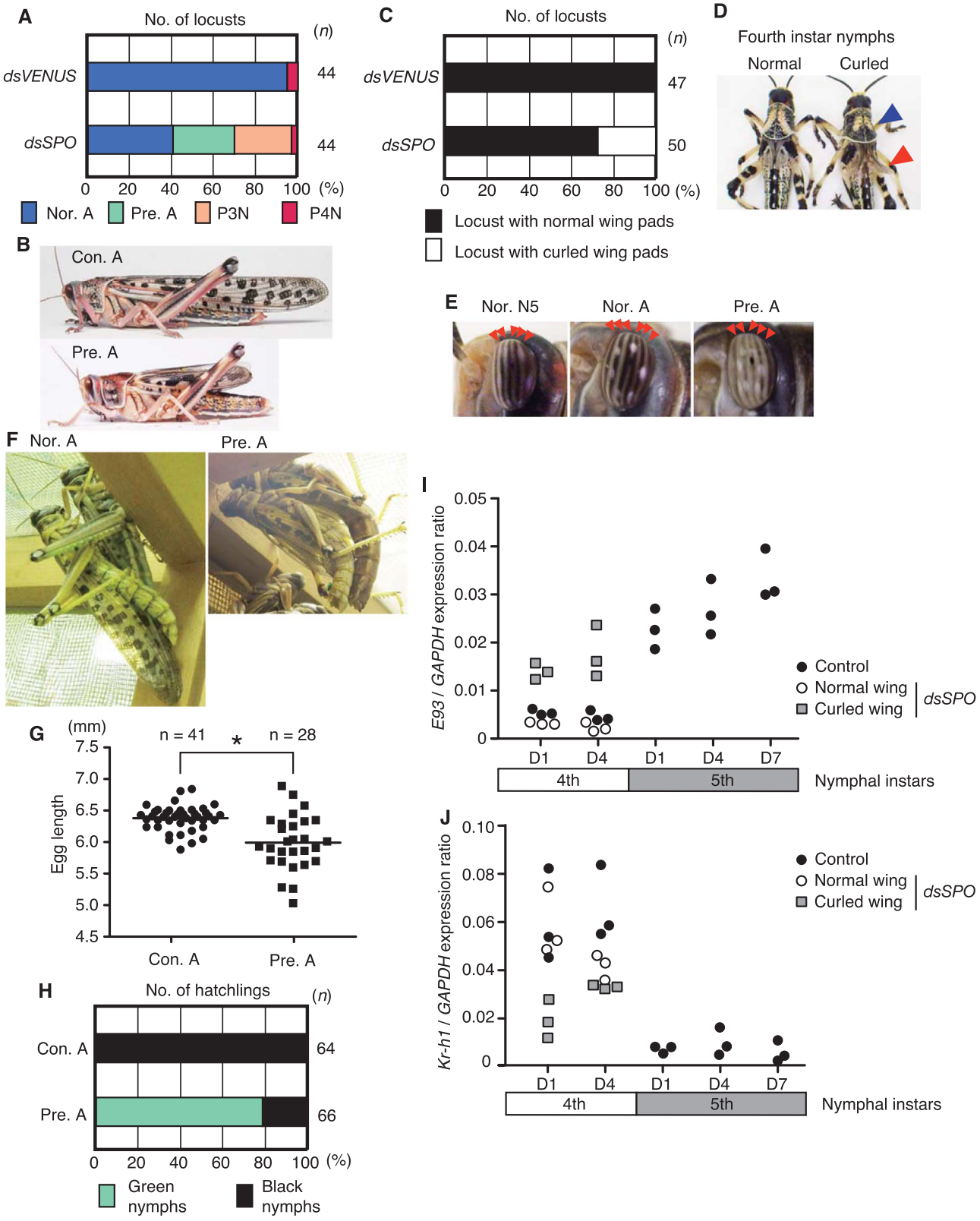

Fig. 3. Precocious adults induced after *dsSPO* injections at the third instar and their characteristics in *S. gregaria*. A, Proportions of nymphs showing the indicated types of developmental fate after injections with *dsSPO* or *dsVENUS* at the third instar. The numbers of test locusts are indicated to the right of the panel. B, A typical normal (top) and precocious adult (bottom). C, Proportions of locusts with normal and curled wing pads after injections with *dsSPO* or *dsVENUS* at the third nymphal instar. The numbers of test locusts are indicated to the right of the panel. D, Typical locusts with normal (left) and curled wing pads (right). Blue and red arrowheads indicate white markings on the pronotum and curled wing pads, respectively. E, Eye stripes (red arrowheads) in a normal fifth instar nymph, and a normal and a precocious adult. F, Mounting behavior of normal and precocious male adults. G, Lengths of eggs laid by control and precocious adults. Each datum point is an average length of ten eggs from the same egg pod. The numbers of test egg pods are indicated above the panel. Bars represent the mean values of the average egg lengths. H, Proportions of green and black hatchlings derived from control and precocious adults. The numbers of test hatchings are indicated to the right of the panel. I, J, Temporal *E93* and *Kr-h1* mRNA expression patterns in nymphs with curled and normal wing pads after injections of *dsSPO* at the third instar as determined by qRT-PCR. Expression of *E93* (I) and *Kr-h1* (J) in the thoracic cuticle of *dsSPO*-injected fourth and non-treated fourth and fifth instar nymphs are compared. *GAPDH* was used as a reference gene for normalization. Each datum point represents an individual. Abbreviations: Nor. A, normal-looking adult; Pre. A, precocious adult; P3N, permanent third instar nymph; P4N, permanent fourth instar nymph; Con. A, control (*dsVENUS*-injected) adult.

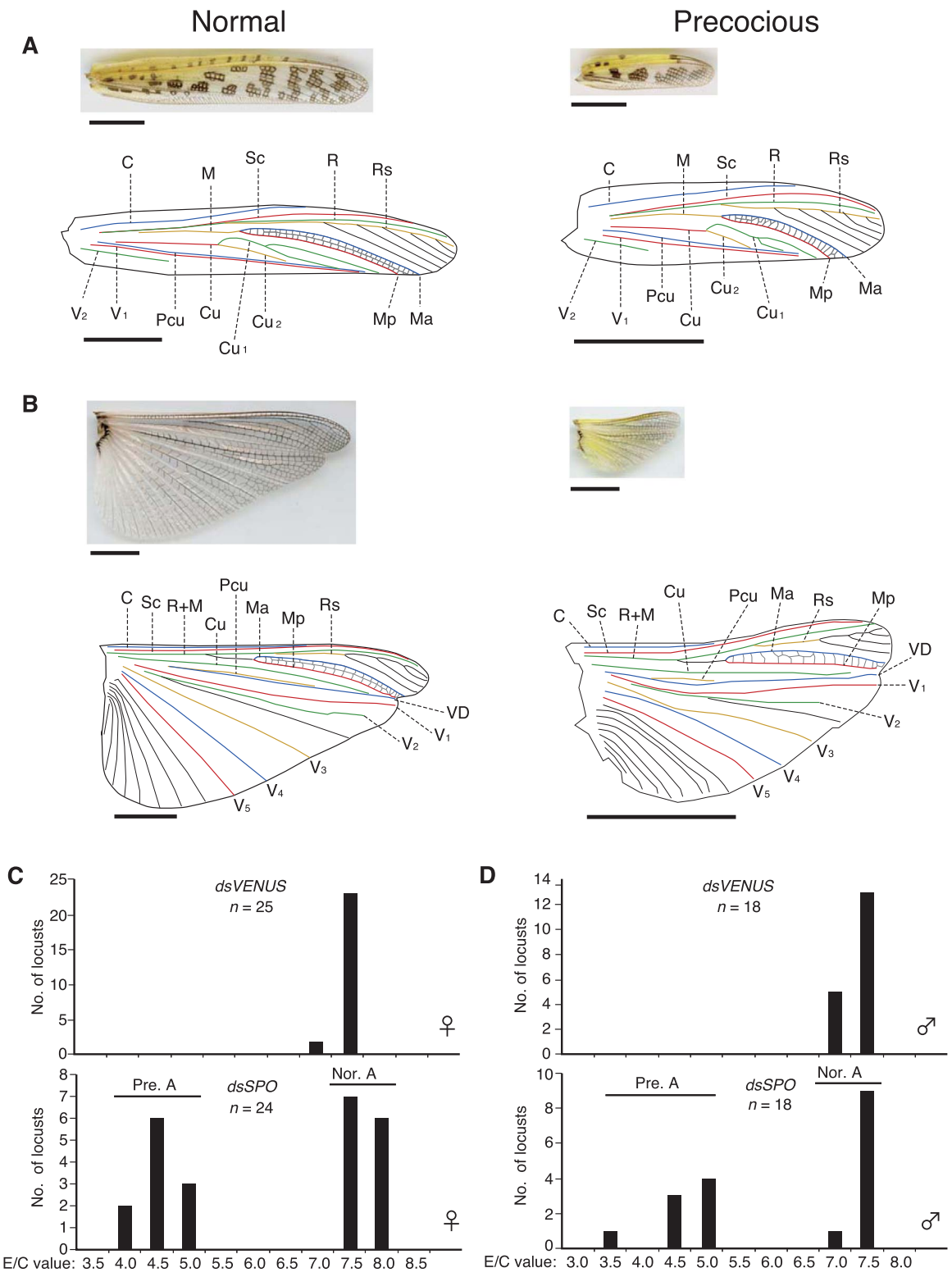

Fig. 4. Comparison of normal and precocious adult wings. A, Photographs and diagrams showing typical normal (left) and precocious (right) forewings. The precocious adult wings were obtained from sexually mature males with yellow body coloration reared under crowded conditions. The main veins were highlighted with colored line. Scale bar = 1 cm. B, Photographs and diagrams showing typical normal (left) and precocious (right) hindwings. The main veins were highlighted with colored lines. Scale bar = 1 cm. C and D, Frequency distributions of E/C (E, forewing length; C, maximum head width) values for adults injected with *dsVENUS* or *dsSPO* at the third instar. Abbreviations: Pc, precosta; C, costa; Sc, subcosta; R, radius; Rs, radial sector; M, media; Ma, anterior media; Mp, posterior media; I, intercalate (false) vein; Cu, cubitus; Pcu, postcuitus; VD, dividing vein; V, vannal vein; Pre. A, precocious adult; Nor. A, normal-looking adult.

fourth instar developed a light-colored face at the fifth instar (Fig. 5A). Such coloration is similar to that observed in normal adults. In contrast, the faces of *dsVENUS*-injected controls were covered with black patterns as typically observed in crowd-reared normal last instar nymphs of this locust. Most of the nymphs with a light-colored face were adultoids (Fig. 5B).

Some nymphs injected with *dsSPO* at the fourth instar failed to leave the exuvia at the following ecdysis (Fig. 5C). The other individuals

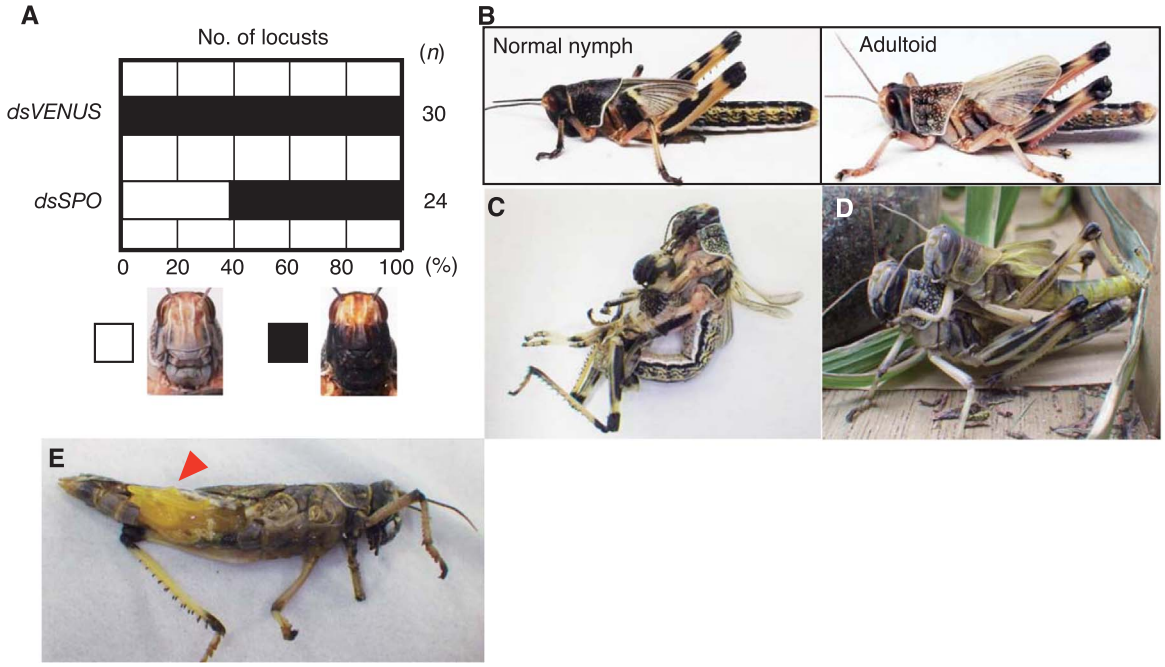

Fig. 5. Adultoids induced after *dsSPO* injections at the fourth instar and their characteristics in *S. gregaria*. A, Proportions of fifth instar locusts with light- (white bar) and dark-colored (black bar) faces after injections with *dsVENUS* or *dsSPO* at the fourth instar. The numbers of test locusts are indicated to the right of the panel. Photographs show a light-colored (left) and dark-colored (right) face. B, Photographs showing a normal fifth instar nymph and an adultoid. C, Photograph of a nymph that failed to escape from the exuvia after *dsSPO* injections at the fourth instar. D, Photograph showing mounting behavior of a male adultoid. E, Photograph of a female adultoid with eggs. The abdomen was cut open to expose eggs. Red arrowhead indicates the eggs.

either became adultoids at the fifth instar or emerged as normal-looking adults after the fifth instar. They were reared separately to observe changes in body color and behavior related to reproduction. At 2 weeks after the last molt, the male adultoids turned yellow (n = 4), as observed for normal-looking male adults (n = 4), and they started mounting on female adultoids (n = 5; Fig. 5D). However, unlike the normal-looking adults, none of the female adultoids laid eggs, although they developed eggs in the ovaries (Fig. 5E). The results indicated that the adultoids obtained after *dsSPO* injections apparently acquired some morphological, behavioral, and reproductive characteristics observed for normal adults.

3.7. Effect of JH injection on the induction of adultoids

The hemolymph JH titer was measured for non-treated fourth (penultimate) instar nymphs. It gradually increased from day 1 onward and peaked at day 4 (Fig. 6A). *Kr-h1* expression, which is expected to increase in accordance with the JH titer (Lozano and Belles, 2011), increased at day 3 of the fourth instar in both *dsVENUS*- and *dsSPO*-injected nymphs. However, part of the *dsSPO*-injected nymphs that showed delayed development had very low *Kr-h1* expression levels at days 7 and 8 (Fig. 6A, red arrowheads). These results suggested that a high JH titer around the peak 20E titer promotes a nymphal-nymphal molt, whereas a low JH titer around this peak induces a molt to adultoid stage. To test this possibility, fourth instar nymphs were first injected with *dsSPO* and with JH or oil (as controls) six days later. As shown in Fig. 6B, no adultoids appeared in the JH-injected nymphs, whereas 20% of oil-injected control nymphs developed into adultoids.

4. Discussion

The present study demonstrated that RNAi of *SPO* in *S. gregaria* delayed nymphal development and interfered with molting, particularly when *dsSPO* was injected into late instar nymphs. These findings are difficult to reconcile with the observations reported by Marchal et al. (2011). We confirmed that *SPO* knockdown at the fifth nymphal instar significantly delayed metamorphosis in another strain of *S. gregaria* (Fig. S2), suggesting that the phenotypes observed in the present study are probably not strain-specific. The hemolymph 20E titers measured during the last nymphal instar using our titration system (Fig. S3) were roughly comparable to those reported by Marchal et al. (2011). The discrepancies in the results of both studies might be related to the fact that Marchal et al. (2011) simultaneously knocked down *SPO* and *PHM*, which functions downstream of *SPO* in 20E synthesis. This treatment might have triggered a feedback mechanism by which the treated locusts could metamorphose without delay. It would be interesting to explore the possible mechanism underlying the seemingly normal metamorphosis without a definite ecdysteroid titer peak in this locust.

The present study revealed that *SPO* knockdown in *S. gregaria* at a certain instar induced precocious metamorphosis (Table S2). To our knowledge, this is the first report of knockdown of a *Halloween* gene inducing such effect in an insect. Precocious metamorphosis is sometimes induced by disturbance of JH production in insects (Daimon et al., 2012; Minakuchi et al., 2008). In the migratory locust, *Locusta migratoria,* nymphs also metamorphose into precocious adults if treated with precocene, an ageratochromene that selectively destroys the corpus allatum, the organ producing JH (Fridman-Cohen and Pener, 1980).

In *D. melanogaster* and *B. mori*, artificially reduced ecdysteroid production induces precocious pupation, although the underlying mechanism is unknown (Bialecki et al., 2002; Danielsen et al., 2016; Kadono-Okuda et al., 1987). In the present study, the 20E level in *dsSPO*-treated *S. gregaria* nymphs showed a delayed, low peak at day 5 of the fourth instar. Because nymphs that delayed molting had reduced *Kr-h1* expression, we injected JH into *dsSPO*-treated nymphs at day 6 to test the possible involvement of JH in this phenomenon. As a result, we observed that JH injection completely inhibited the appearance of adultoids or precocious metamorphosis. This result may suggest that the *dsSPO*-induced delay in ecdysis causes a low JH titer along with the low 20E peak in the nymphs, inducing precocious metamorphosis. Similar hormonal modifications might

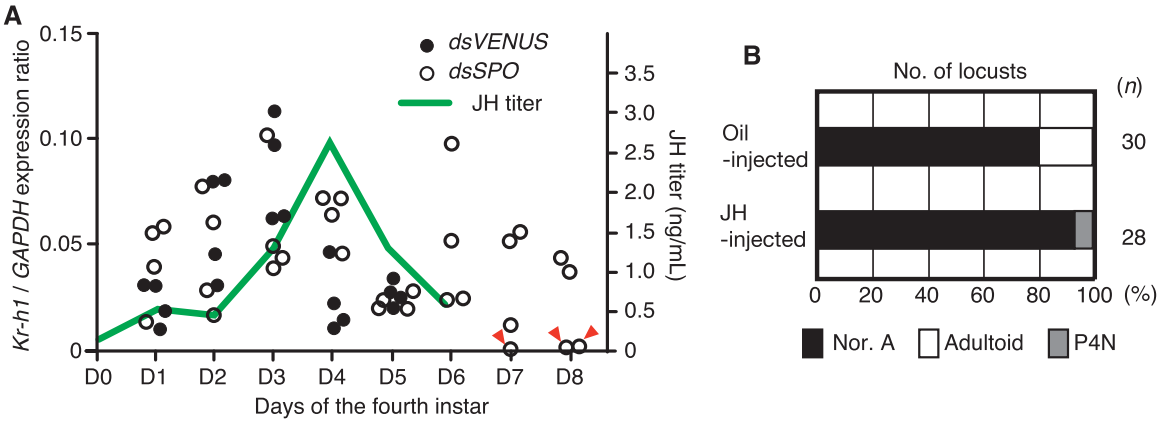

Fig. 6. Effects of *dsSPO* and JH injections on development in *S. gregaria*. A, Temporal *Kr-h1* mRNA expression patterns in the thoracic cuticle of fourth instar nymphs after injections of *dsVENUS* or *dsSPO* at day 0 of this instar as determined by qRT-PCR. *GAPDH* was used as a reference gene for normalization. Each datum point represents an individual. Red arrowheads indicate extremely low levels of *Kr-h1* expression in *dsSPO*-injected nymphs. The JH titer profile was determined by LC-MS for non-treated penultimate instar nymphs only. The JH titers for days 2, 3, 4, 5, and 6 are mean values of two pooled samples. B, Proportions of normal-looking adults, adultoids, and permanent fourth instar nymphs in the *dsSPO*-injected locusts after injection of JHIII mixed with peanut oil (100 μg in 2 μL) or peanut oil alone (control) at day 6 of the fourth instar. All nymphs were injected with *dsSPO* at day 0 of the fourth instar. Two nymphs never molted to the next instar after JH injection, and died. The numbers of test locusts are indicated to the right of the panel. Abbreviations: Nor. A, normal-looking adult; P4N, permanent fourth instar nymph.

have occurred in precociously metamorphosed *D. melanogaster* and *B. mori* in the above reports.

However, this hypothesis may not explain the mechanism by which precocious metamorphosis was induced after injections of *dsSPO* at the third nymphal instar in *S. gregaria*. In this case, no individual metamorphosed at the following molt. Precocious adults appeared only after the second molt (Table S2). Although the 20E and JH titers during the third and fourth instars should be determined, the mechanisms of precocious metamorphosis induced after *SPO* knockdown at the third and fourth instars might be different. In *B. mori*, precocious metamorphic molt or pupation can be induced by extirpating the corpora allata (Bounhiol, 1937; Kim, 1939) or by artificially elevating JH esterase activity (Tan et al., 2005) in mid or late instar nymphs, indicating the pivotal role of JH in metamorphosis. In the linden bug *Pyrrhocoris apterus*, knockdown of certain genes downstream of JH signaling at mid or late nymphal instars induced signs of precocious metamorphosis, whereas those at early nymphal instars failed to produce precocious metamorphosis (Smykal et al., 2014). Daimon et al. (2015) have reported that pupation is inhibited during the first two larval instars even after knockout of the genes responsible for JH synthesis and the receptor system in *B. mori*, suggesting that a JH-independent mechanism inhibits metamorphic molting in the early larval instars. A similar phenomenon has been reported for *L. migratoria* after treatment of the eggs with precocene. In this locust, precocious metamorphosis is inhibited during the first two nymphal instars in spite of the precocene-induced degeneration of the embryonic corpora allata (Aboulafia-Baginsky et al., 1984).

In addition to 20E and JH, metamorphosis is regulated by various other factors, which are sometimes influenced by these hormones. For example, in the cockroach *B. germanica,* E93 and Kr-h1 are closely associated with metamorphosis. In the penultimate instar, the anti-metamorphic transcription factor Kr-h1 induced by JH represses expression of E93, a key metamorphosis gene (Belles and Santos, 2014; Ureña et al., 2014). In the last instar, JH production ceases, which suppresses *Kr-h1* expression, resulting in *E93* expression and metamorphosis (Belles and Santos, 2014). In *S. gregaria*, the *dsSPO*-injected fourth instar nymphs with curled wing pads, which underwent precocious metamorphosis after the following molt, showed temporal *E93* and *Kr-h1* expression patterns similar to those observed in the last nymphal instar. In contrast, nymphs with normal-looking wing pads, which underwent a nymphal-nymphal molt, exhibited a reversed pattern similar to that of non-treated penultimate instar nymphs. These results suggest that fourth instar nymphs with curled wing pads are physiologically similar to nymphs at the last instar.

Knockdown of *SPO* in *S. gregaria* affected various other phenotypic traits, such as wing length. Some of the treated individuals developed short wings that looked like those of the short-winged morph known for *L. migratoria,* which shows wing dimorphism in laboratory colonies (Nishide and Tanaka, 2013a, 2013b; Tanaka and Nishide, 2012). *S. gregaria* is a long-winged species, and no short-wing morph has been reported to date. The short wings observed after *SPO* knockdown in *S. gregaria* showed abbreviated venation, as often observed in wing-polyphenic insects (Shimizu and Masaki, 1993; Solbreck, 1986; Tanaka et al., 2001; Tanaka and Wolda, 1987). The variation in wing length observed in this study showed a bimodal pattern, representing locusts with short wings and those with normal-looking wings. The production of short wings upon knockdown of an ecdysteroid-related gene might suggest a phylogenetic or evolutionary potential to develop wing dimorphism in this locust. Further studies are necessary to understand the mechanism determining wing length in this species.

Both precocious adults and adultoids displayed several adult characteristics when reared under crowded conditions; males developed a bright yellow body color, a symptom of sexual maturation in this species under crowded conditions (Nishide and Tanaka, 2012; Pener, 1991), and mounted on sexually mature females. While the adultoid females developed eggs in the ovaries, none of these eggs were laid. In contrast, precocious female adults with short wings laid eggs that were significantly smaller than those laid by control females. From these eggs, relatively small, green hatchlings appeared. Green hatchlings are usually produced only by solitarious females living at a low population density or by isolation-reared females in the laboratory (Hunter-Jones, 1958). At a high population density or under crowded conditions, hatchlings are relatively large and black. The black color is probably controlled by corazonin (Crz), because in an albino strain of this locust harboring a defective Crz receptor gene, all hatchlings are green, with no black patterns, even under crowded conditions (Sugahara et al., 2017). *S. gregaria* might provide an excellent system to explore the functions of ecdysteroids, Crz, and possibly JH in the control of progeny size and characteristics.

In conclusion, knockdown of *SPO* in *S. gregaria*, was found to interfere with molting and to induce precocious metamorphosis in this locust. Precocious adults exhibited some adult characteristics including yellowing, mating behavior, and oviposition, whereas adultoids developed eggs, but did not lay the eggs. Based on preliminary data on hemolymph ecdysteroid and JH titers and *Kr-h1* expression, the

temporal relationship of these hormones was hypothesized to be important in the induction of precocious adults, and this hypothesis was supported by the fact that administration of JH to *dsSPO*-injected nymphs prevented the production of precocious adults. Further studies on the functions of E93 and Kr-h1 and determination of the JH and ecdysteroid titers in *dsSPO*-injected nymphs will be necessary to better understand the molecular mechanism of molting and metamorphosis in *S. gregaria*.

Acknowledgments

We wish to thank Ms. Hiroko Ikeda, Ms. Masako Higuchi, Ms. Utako Takano, and Ms. Harumi Murata for assistance with the maintenance of locust colonies, and Ms. Takayo Nakakura for assistance with part of the experimental work. Thanks are also due to Dr. Takumi Kayukawa who advised us on the molecular mechanism of insect development and constructive comments on an early draft of the paper. We are also grateful to Emer. Prof. Kenji Kiguchi of Shinshu University, who kindly shared with us his unpublished information about hormonal control of metamorphosis in silkworms. This work was supported in part by a JSPS Kakenhi Grant (number 15J08228) and JSPS Research Fellowships for Young Scientists to RS. We thank Mr. Kameo Tsukada and Mr. Hirokazu Tomiyama of the Field Management Section of NARO for growing the grass used in this study.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ydbio.2017.07.007.

References

Aboulafia-Baginsky, N., Pener, M.P., Staal, G.B., 1984. Chemical allatectomy of late *Locusta* embryos by a synthetic precocene and its effect on hopper morphogenesis. J. Insect Physiol., 30. http://dx.doi.org/10.1016/0022-1910(84)90057-X.

Belles, X., Santos, C.G., 2014. The MEKRE93 (Methoprene tolerant-Krüppel homolog 1-E93) pathway in the regulation of insect metamorphosis, and the homology of the pupal stage. Insect Biochem. Mol. Biol. 52, 60–68. http://dx.doi.org/10.1016/j.ibmb.2014.06.009.

Bialecki, M., Shilton, A., Fichtenberg, C., Segraves, W.A., Thummel, C.S., 2002. Loss of the ecdysteroid-inducible E75A orphan nuclear receptor uncouples molting from metamorphosis in *Drosophila*. Dev. Cell 3, 209–220. http://dx.doi.org/10.1016/S1534-5807(02)00204-6.

Bounhiol, J.J., 1937. La métamorphose des insectes sereit inhibée dans leur jeune age par les corpora allata. C. R. Soc. Biol. 126, 1189–1191.

Daimon, T., Kozaki, T., Niwa, R., Kobayashi, I., Furuta, K., Namiki, T., Uchino, K., Banno, Y., Katsuma, S., Tamura, T., Mita, K., Sezutsu, H., Nakayama, M., Itoyama, K., Shimada, T., Shinoda, T., 2012. Precocious metamorphosis in the juvenile hormone-deficient mutant of the silkworm, *Bombyx mori*. PLoS Genet., 8. http://dx.doi.org/10.1371/journal.pgen.1002486.

Daimon, T., Uchibori, M., Nakao, H., Sezutsu, H., Shinoda, T., 2015. Knockout silkworms reveal a dispensable role for juvenile hormones in holometabolous life cycle. Proc. Natl. Acad. Sci. USA 112, E4226–E4235. http://dx.doi.org/10.1073/pnas.1506645112.

Danielsen, E.T., Moeller, M.E., Yamanaka, N., Ou, Q., Laursen, J.M., Soenderholm, C., Zhuo, R., Phelps, B., Tang, K., Zeng, J., Kondo, S., Nielsen, C.H., Harvald, E.B., Faergeman, N.J., Haley, M.J., O'Connor, K.A., King-Jones, K., O'Connor, M.B., Rewitz, K.F., 2016. A *Drosophila* genome-wide screen identifies regulators of steroid hormone production and developmental timing. Dev. Cell 37, 558–570. http://dx.doi.org/10.1016/j.devcel.2016.05.015.

De Loof, A., Boerjan, B., Ernst, U.R., Schoofs, L., 2012. The mode of action of juvenile hormone and ecdysone: towards an epi-endocrinological paradigm? Gen. Comp. Endocrinol. 188, 35–45. http://dx.doi.org/10.1016/j.ygcen.2013.02.004.

Enya, S., Ameku, T., Igarashi, F., Iga, M., Kataoka, H., Shinoda, T., Niwa, R., 2014. A Halloween gene *noppera-bo* encodes a glutathione S-transferase essential for ecdysteroid biosynthesis via regulating the behaviour of cholesterol in *Drosophila*. Sci. Rep. 4, 6586. http://dx.doi.org/10.1038/srep06586.

Fridman-Cohen, S., Pener, M.P., 1980. Precocenes induce effect of juvenile hormone excess in *Locusta migratoria*. Nature 286, 711–713. http://dx.doi.org/10.1038/286711a0.

Furuta, K., Ichikawa, A., Murata, M., Kuwano, E., Shinoda, T., Shiotsuki, T., 2013. Determination by LC-MS of juvenile hormone titers in hemolymph of the silkworm, *Bombyx mori*. Biosci. Biotechnol. Biochem. 77, 988–991. http://dx.doi.org/10.1271/bbb.120908.

Hunter-Jones, P., 1958. Laboratory studies on the inheritance of phase characters in locusts. Anti-Locust Bull. 29, 1–32.

Jia, S., Wan, P.J., Zhou, L.T., Mu, L.L., Li, G.Q., 2013. Knockdown of a putative Halloween gene *Shade* reveals its role in ecdysteroidogenesis in the small brown planthopper *Laodelphax striatellus*. Gene 531, 168–174. http://dx.doi.org/10.1016/j.gene.2013.09.034.

Kadono-Okuda, K., Kuwano, E., Eto, M., Yamashita, O., 1987. Inhibitory action of an imidazole compound on ecdysone synthesis in prothoracic glands of the silkworm, *Bombyx mori*. Dev. Growth Differ. 29, 527–533. http://dx.doi.org/10.1111/j.1440-169X.1987.00527.x.

Kim, S.B., 1939. Observations on the corpus allatum hormone in *Bombyx mori*. J. Sericult. Sci. 10, 86–97.

Lenaerts, C., Van Wielendaele, P., Peeters, P., Vanden Broeck, J., Marchal, E., 2016. Ecdysteroid signalling components in metamorphosis and development of the desert locust, *Schistocerca gregaria*. Insect Biochem. Mol. Biol. 75, 10–23. http://dx.doi.org/10.1016/j.ibmb.2016.05.003.

Lozano, J., Belles, X., 2011. Conserved repressive function of Krüppel homolog 1 on insect metamorphosis in hemimetabolous and holometabolous species. Sci. Rep. 1, 163. http://dx.doi.org/10.1038/srep00163.

Marchal, E., Badisco, L., Verlinden, H., Vandersmissen, T., Van Soest, S., Van Wielendaele, P., Vanden Broeck, J., 2011. Role of the *Halloween* genes, *Spook* and *Phantom* in ecdysteroidogenesis in the desert locust, *Schistocerca gregaria*. J. Insect Physiol. 57, 1240–1248. http://dx.doi.org/10.1016/j.jinsphys.2011.05.009.

Marchal, E., Verlinden, H., Badisco, L., Van Wielendaele, P., Vanden Broeck, J., 2012. RNAi-mediated knockdown of *Shade* negatively affects ecdysone-20-hydroxylation in the desert locust, *Schistocerca gregaria*. J. Insect Physiol. 58, 890–896. http://dx.doi.org/10.1016/j.jinsphys.2012.03.013.

Minakuchi, C., Namiki, T., Yoshiyama, M., Shinoda, T., 2008. RNAi-mediated knockdown of juvenile hormone acid O-methyltransferase gene causes precocious metamorphosis in the red flour beetle *Tribolium castaneum*. FEBS J. 275, 2919–2931. http://dx.doi.org/10.1111/j.1742-4658.2008.06428.x.

Nakagawa, Y., Henrich, V.C., 2009. Arthropod nuclear receptors and their role in molting. FEBS J. 276, 6128–6157. http://dx.doi.org/10.1111/j.1742-4658.2009.07347.x.

Nishide, Y., Tanaka, S., 2012. Yellowing, morphology and behaviour in sexually mature gynandromorphs of the desert locust *Schistocerca gregaria*. Physiol. Entomol. 37, 379–383. http://dx.doi.org/10.1111/j.1365-3032.2012.00854.x.

Nishide, Y., Tanaka, S., 2013a. The occurrence in the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae), of a short-winged morph with no obvious fitness advantages over the long-winged morph. Eur. J. Entomol. 110, 577–583. http://dx.doi.org/10.14411/eje.2013.078.

Nishide, Y., Tanaka, S., 2013b. Wing dimorphism in the migratory locust, *Locusta migratoria*: differentiation of wing morph and phase polyphenism. Entomol. Sci. 16, 421–431. http://dx.doi.org/10.1111/ens.12023.

Niwa, R., Niwa, Y.S., 2014. Enzymes for ecdysteroid biosynthesis: their biological functions in insects and beyond. Biosci. Biotechnol. Biochem. 78, 1283–1292. http://dx.doi.org/10.1080/09168451.2014.942250.

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., Shinoda, T., 2010. Non-molting glossy/shroud encodes a short-chain dehydrogenase/reductase that functions in the "Black Box" of the ecdysteroid biosynthesis pathway. Development 137, 1991–1999. http://dx.doi.org/10.1242/dev.045641.

Pener, M.P., 1991. Locust phase polymorphism and its endocrine relations. Adv. Insect Physiol. 23, 1–79.

Shimizu, T., Masaki, S., 1993. Injury causes microptery in the ground cricket, *Dianemobius fascipes*. J. Insect Physiol. 39, 1021–1027.

Shiotsuki, T., Hua, Y., Tsugane, T., Gee, S., Hammock, B.D., 2005. Optimization of an enzyme-linked immunosorbent assay for ecdysteroids. J. Insect Biotechnol. Sericol. 4, 1–4.

Smykal, V., Daimon, T., Kayukawa, T., Takaki, K., Shinoda, T., Jindra, M., 2014. Importance of juvenile hormone signaling arises with competence of insect larvae to metamorphose. Dev. Biol. 390, 221–230. http://dx.doi.org/10.1016/j.ydbio.2014.03.006.

Solbreck, C., 1986. Wing and flight muscle polymorphism in a lygaeid bug, *Horvathiolus gibbicollis*: determinants and life history consequences. Ecol. Entomol. 11, 435–444.

Sugahara, R., Mon, H., Lee, J.M., Kusakabe, T., 2014. Middle region of FancM interacts with Mhf and Rmi1 in silkworms, a species lacking the Fanconi anaemia (FA) core complex. Insect Mol. Biol. 23, 185–198. http://dx.doi.org/10.1111/imb.12072.

Sugahara, R., Jouraku, A., Nakakura, T., Kusakabe, T., Yamamoto, T., Shinohara, Y., Miyoshi, H., Shiotsuki, T., 2015a. Two adenine nucleotide translocase paralogues involved in cell proliferation and spermatogenesis in the silkworm *Bombyx mori*. PLoS One 10, e0119429. http://dx.doi.org/10.1371/journal.pone.0119429.

Sugahara, R., Saeki, S., Jouraku, A., Shiotsuki, T., Tanaka, S., 2015b. Knockdown of the corazonin gene reveals its critical role in the control of gregarious characteristics in the desert locust. J. Insect Physiol. 79, 80–87. http://dx.doi.org/10.1016/j.jinsphys.2015.06.009.

Sugahara, R., Tanaka, S., Jouraku, A., Shiotsuki, T., 2016. Functional characterization of the corazonin-encoding gene in phase polyphenism of the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae). Appl. Entomol. Zool. 51, 225–232. http://dx.doi.org/10.1007/s13355-015-0391-2.

Sugahara, R., Tanaka, S., Jouraku, A., Shiotsuki, T., 2017. Two types of albino mutants in desert and migratory locusts are caused by gene defects in the same signaling pathway. Gene 608, 41–48. http://dx.doi.org/10.1016/j.gene.2017.01.022.

Tan, A., Tanaka, H., Tamura, T., Shiotsuki, T., 2005. Precocious metamorphosis in transgenic silkworms overexpressing juvenile hormone esterase. Proc. Natl. Acad. Sci. USA 102, 11751–11756. http://dx.doi.org/10.1073/pnas.0500954102.

Tanaka, S., Nishide, Y., 2012. First record of the occurrence and genetics of a short-

winged morph in the migratory locust, *Locusta migratoria* (Orthoptera: Acrididae). J. Orthoptera Res. 21, 169–174.
Tanaka, S., Nishide, Y., 2013. Behavioral phase shift in nymphs of the desert locust, *Schistocerca gregaria*: special attention to attraction/avoidance behaviors and the role of serotonin. J. Insect Physiol. 59, 101–112. http://dx.doi.org/10.1016/j.jinsphys.2012.10.018.
Tanaka, S., Wolda, H., 1987. Seasonal wing length dimorphism in a tropical seed bug: ecological significance of the short-winged form. Oecologia 73, 559–565. http://dx.doi.org/10.1007/BF00379416.
Tanaka, S., Yagi, S., 1997. Evidence for the involvement of a neuropeptide in the control of body color in the desert locust, *Schistocerca gregaria*. Jpn. J. Entomol. 65, 447–457.
Tanaka, S., Katagiri, C., Arai, T., Nakamura, K., 2001. Continuous variation in wing length and flight musculature in a tropical field cricket, *Teleogryllus derelictus*: implication for the evolution of wing dimorphism. Entomol. Sci. 4, 195–208.
Tanaka, S., Harano, K., Nishide, Y., Sugahara, R., 2016. The mechanism controlling phenotypic plasticity of body color in the desert locust: some recent progress. Curr. Opin. Insect Sci. 17, 10–15. http://dx.doi.org/10.1016/j.cois.2016.05.011.
Truman, J.W., Riddiford, L.M., 2002. Endocrine insights into the evolution of metamorphosis in insects. Annu. Rev. Entomol. 47, 467–500.
Ureña, E., Manjón, C., Franch-Marro, X., Martín, D., 2014. Transcription factor E93 specifies adult metamorphosis in hemimetabolous and holometabolous insects. Proc. Natl. Acad. Sci. USA 111, 7024–7029. http://dx.doi.org/10.1073/pnas.1401478111.
Ureña, E., Chafino, S., Manjón, C., Franch-Marro, X., Martín, D., 2016. The occurrence of the holometabolous pupal stage requires the interaction between E93, Krüppel-Homolog 1 and Broad-Complex. PLoS Genet. 12, 1–24. http://dx.doi.org/10.1371/journal.pgen.1006020.
Uvarov, B., 1966. Grasshoppers and Locusts: a handbook of general acridology. Vol. 1. Anatomy, Physiology, Development, Phase Polymorphism, Introduction to Taxonomy. Cambridge Cent. Overseas Pest Res. 1.
Yoshiyama, T., Namiki, T., Mita, K., Kataoka, H., Niwa, R., 2006. Neverland is an evolutionally conserved Rieske-domain protein that is essential for ecdysone synthesis and insect growth. Development 133, 2565–2574. http://dx.doi.org/10.1242/dev.02428.