Rectal sac distention is induced by 20-hydroxyecdysone in the pupa of Bombyx mori

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4 **Rectal sac distention is induced by 20-hydroxyecdysone in the pupa of *Bombyx mori***

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22

1   **Abstract**

2   Holometabolous insects do not excrete but store metabolic wastes during the pupal

3   period. The waste is called meconium and is purged after adult emergence. Although the

4   contents of meconium are well-studied, the developmental and physiological regulation of

5   meconium accumulation is poorly understood. In *Bombyx mori*, meconium is accumulated in

6   the rectal sac; thereby, the rectal sac distends at the late pupal stage. Here, we show that rectal

7   sac distention occurs between 4 and 5 days after pupation. The distention is halted by

8   brain-removal just after larval-pupal ecdysis but not by brain-removal one day after pupation. In

9   the pupae, brain-removal just after ecdysis kept the hemolymph ecdysteroid titer low during

10   early and mid-pupal stages. An injection of 20-hydroxyecdysone (20E) evoked the distention

11   that was halted by brain-removal in a dose-dependent manner. Therefore, brain-removal caused

12   the lack of ecdysteroid, and rectal sac distention did not appear in the brain-removed pupae

13   because of the lack of ecdysteroid. We conclude that rectal sac distention is one of the

14   developmental events regulated by 20E during the pupal period in *B. mori*.

15

16   **Keywords:** excretory system, ecdysone, brain, metamorphosis, meconium

17

1. Introduction

Insects are classified as ametabolous, hemimetabolous, or holometabolous according to the type of postembryonic development that occurs. Holometabolous insects undergo an elaborate developmental sequence called metamorphosis, and their excretory system is critical for development. The insects stop feeding at the end of the last larval instar and then become pupae to start adult development without feeding and excreting. They store metabolic wastes as meconium during the pupal period and then excrete it after adult emergence. The major components of meconium are uric acid (Brown, 1938) and nitrogenous substances (Levenbook et al., 1971). In addition, the meconium of *Manduca sexta* contains degraded ecdysteroids such as 3-epi-20-hydroxyecdysone (Thompson et al., 1974), 20-hydroxyecdysonoic acid, and 3-α-epi-20, 26-dihydroxyecdysone (Warren and Gilbert, 1986). The larvae purge their gut contents at the end of the feeding period (Kiguchi, 1985), and this purge is regulated by ecdysteroid (Nagata et al., 1987). Ablation of the rectal sac from newly ecdysed pupae disturbs adult eclosion (Dedos and Fugo, 1999). Although the excretory system is important for insect development, the system has not been elucidated well.

Judy and Gilbert (1970a, b) reported that the morphological change of the alimentary canal in *Hyalophora cecropia* was influenced by the administration of juvenile hormone. In *B. mori*, treatment with one of the juvenile hormone analogs, fenoxycarb, and 20-hydroxyecdysone (20E) resulted in the disruption of rectal development (Dedos and Fugo, 1999). The rectal sac started to increase in size 120 h after pupation, but the factor inducing the increase has not been identified. Thus, developmental and physiological regulation of the excretory system in holometabolous insects is still unknown.

In the present study, we examine the developmental change and hormonal regulation

1 of rectal sac distention in *B. mori* as the model of the excretory system. We show that distention

2 occurred between 4 and 5 days after pupation. The distention was halted by brain-removal just

3 after larval-pupal ecdysis and was evoked by 20E administration. Thus, the occurrence of rectal

4 sac distention was developmentally controlled by 20E titer.

5

6 **2. Materials and methods**

7 *2.1 Animals*

8   *B. mori* (Kinshu $\times$ Showa) larvae were reared on an artificial diet (Silkmate 2M,

9 Nihon Nosan Kougyo, Yokohama, Japan) at $25 \pm 1°C$ under a 12 h light: 12 h dark

10 photoperiodic regime. The day of pupation was designated as day 0 (P0). One day after

11 pupation and 2 – 8 days after pupation were designated as stages P1 and P2 – P8, respectively.

12 In this study, the pupae just after larval-pupal ecdysis were described as white pupae; this stage

13 was designated as WP.

14

15 *2.2 Hormones*

16   α-ecdysone and 20E were obtained from Sigma (St Louis, MO) and dissolved in

17 ethanol and distilled water, respectively. [^{3}H]-ecdysone (Perkin Elmer, Boston, MA) was

18 dissolved in borate buffer (100 mM boric acid, 50 mM borax, 60 mM NaCl). 20E was diluted

19 with insect Ringer's solution (128 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) for injections.

20

21 *2.3 Operation and observations*

22   Pupal brains were removed at WP and P0 – P2. For operational control, a hole was

23 made in the head of each pupa and the brain was left intact. The pupae that were brain-removed

1 at WP were injected with 10 μl of insect Ringer's solution or 20E solution. The wound made by

2 the operation or injection was sealed with melted paraffin wax. The degree of the rectal sac

3 distention was described as follows: no distention, in which the sac contains little or no

4 meconium; and distention, in which the sac is filled with meconium.

5

6 *2.4. Quantification of ecdysteroid titer*

7 　　Hemolymph was collected from pupae by cutting the dorsal side. Ecdysteroids were

8 extracted from hemolymph and quantified by radioimmunoassay as described previously

9 (Sakurai et al., 1998). Anti-ecdysone antiserum H-22 was obtained from L. I. Gilbert and D. H.

10 S. Horn and used as a capture antibody in the radioimmunoassay (Warren and Gilbert, 1986).

11

12 **3. Results**

13 *3.1. Rectal sacs distended between P4 and P5*

14 　　To examine when the rectal sacs distend, pupae were dissected at P3 – P6. Before P3,

15 the rectal sacs were not observed. The sacs appeared but did not distend at P3 (Fig. 1A, left

16 panel). A distended sac was observed in one of 17 pupae at P4 and in almost all pupae at P5

17 (Fig. 1B, n = 19). At P6, distended rectal sacs were observed in all pupae. These observations

18 suggest that the rectal sac distends between P4 and P5.

19

20 *3.2. Brain-removal halted the distention of the rectal sac*

21 　　Hemolymph titers of prothoracicotropic hormone (PTTH) (Mizoguchi et al., 2001)

22 and bombyxin (Saegusa et al., 1992) are kept high levels during P4 and P5 and the titer of

23 PTTH is the highest during P1 and P2 (Mizoguchi et al., 2001). We examined whether these

1   peptides are responsible for the distention of the sacs by removing the brains from pupae at P0 –

2   P2. The pupae were reared until P6. No distended rectal sacs appeared in the P6 pupae

3   brain-removed at WP, while distended sacs appeared in approximately half of the P6 pupae that

4   had their brains removed at P0 (52 ± 17%) and in all P6 pupae after brain removal at P1 or P2

5   (Fig. 2). The sacs were distended in most control P6 pupae operated on at WP and P0 – P2.

6   Brains of WP pupae and P0 pupae were essential for the distention, but those of P1 or P2 were

7   no longer essential for the distention. When the pupae were removed their brains at WP and

8   replanted the removed brains after washing with insect Ringer's solution, the pupae showed

9   rectal sac distention in 69% of the operated pupae (n = 13). These results indicate that a brain of

10   WP pupa gives sufficient and necessary factor(s) for rectal sac distention.

11

12   *3.3. 20E induced rectal sac distention*

13   Figure 2 implies that the brain of a WP pupa contains an essential factor(s) for the

14   distention. When that factor is PTTH, a lack of ecdysteroid may cause the failure of the

15   distention in P6 pupa that had their brains removed at WP. We injected 20E (0.25 - 3.0 μg/g

16   body weight) to P2 pupae brain-removed at WP. The injected pupae were reared until P8. As

17   shown in Figure 3, 3.0 μg/g body weight 20E induced distention in all injected pupae. Over the

18   range from 0.25 to 3.0 μg/g body weight, 20E induced distention in a dose-dependent manner.

19   Insect Ringer's solution did not induce the distention. This result shows that distention is

20   induced by 20E, indicating that a lack of 20E causes the failure of distention in pupae with

21   brains removed at WP.

22   We examined contributions of juvenile hormone in rectal sac distention by allatectomy

23   in fourth instar larvae. Distended rectal sacs appeared in 93% of allatectomized precocious

1   pupae on day 6 (n=14). The source of juvenile hormone, the corpora allata, was therefore not

2   essential for the distention.

3

4

5   *3.4 Four days after injection with 20E is sufficient time to induce distention*

6   We examined what time period is sufficient to induce the distention caused by 20E.

7   We injected 20E to the brain-removed pupae at P2 and dissected the pupae at P4 – P8.

8   Distended sacs appeared in most pupae at P6 and in all pupae at P8 (Fig. 3B). They did not

9   appear at P4 and appeared in a few pupae at P5. The appearance of the distention after P6

10   indicates that the rectal sac distention is induced four days after 20E-injection.

11

12   *3.5 Ecdysteroid titer from P0 to P6 in the 20E-injected pupae*

13   Figure 3 shows that the rectal sacs were distended at P6 of 20E-injected pupae. We

14   determined the ecdysteroid titer of 20E-injected pupae from P0 to P6. The titers of 20E-injected

15   pupae were 4.36 ± 0.37 μM and 4.44 ± 0.72 μM at 1 and 2 days after injection, respectively

16   (Fig. 4). Then, it decreased sharply to reach the control level. The titer of ecdysteroid decreased

17   at P5 in the 20E-injected pupae. In the control experiment, the ecdysteroid titer was kept at a

18   constant level (1.05 – 1.78 μM, $n = 7 - 8$).

19

20   *3.6 Ecdysteroid elevation was inhibited in a stage specific manner*

21   Brain-removal at WP inhibited rectal sac distention completely, but brain-removal at

22   P1 did not (Fig. 2). We examined whether the difference of inhibition by brain-removal was

23   caused by ecdysteroid. Ecdysteroid was extracted from the P2 pupae with brains removed at

7

1 WP, P0, and P1, and then its amount was measured by radioimmunoassay. The levels of

2 ecdysteroid were 4.20 ± 1.09 μM and 5.62 ± 0.61 μM in the pupae operated at P0 and P1,

3 respectively (Fig. 5). By contrast, the level was 1.05 ± 0.27 μM in the pupae operated at WP.

4 Thus, ecdysteroid elevation was inhibited by brain-removal at WP.

5

6 **4. Discussion**

7 Here, we show that rectal sac distention occurs during P4 – P5. The distention is

8 halted by brain-removal from WP pupae, and distention resumes after 20E injection. The

9 resumption of distention after 20E injection indicates that a lack of ecdysteroid prevents the sac

10 from distending. Ecdysteroid production in the prothoracic gland, an ecdysteroidogenic organ,

11 is activated by PTTH secreted from the brain (Kawakami et al., 1990). Brain-removal causes a

12 lack of PTTH and, thereby, a decrease in the ecdysteroid level. Thus, the brain is essential for

13 distention by regulating ecdysteroidogenesis.

14 The ecdysteroid titer was high during P2 – P4 (Fig. 4) and declines sharply to an

15 undetectable level at the time of eclosion (Mizoguchi et al., 2001). In the pupae that had brains

16 removed and had been injected with insect Ringer's solution, the hemolymph ecdysteroid level

17 was kept at concentrations ranging from 1.05 ± 0.27 to 1.78 ± 0.39 μM during P0 – P6 (Fig. 4).

18 The amount of PTTH was expressed as *Bombyx* unit as in a previous study (Ishizaki et al.,

19 1983). A *Bombyx* unit is defined as the minimum amount of PTTH necessary to induce adult

20 development in more than half of the brain-removed pupae. A *Bombyx* unit of PTTH is

21 equivalent to 110 pg (Kataoka et al., 1987). The PTTH concentration of the newly ecdysed pupa

22 is approximately 100 pg/ml (Mizoguchi et al., 2001), and the newly ecdysed pupae therefore

23 contain enough PTTH in their hemolymph to initiate adult development. However, the

1　brain-removed pupae never initiate adult development in *B. mori* (Kobayashi and Kimura,

2　1958). When the brain-removed pupae were injected with brain extract, they initiate adult

3　development. Figure 5 shows that brain-removal at WP significantly inhibited ecdysteroid

4　elevation and brain-removal at P0 and P1 did not. Therefore, the pupae brain-removed at WP

5　did not contain enough PTTH to activate the prothoracic glands, and the failure of the rectal sac

6　to distend may be due to the lack of PTTH.

7　　　　The higher ecdysteroid level during P2 – P4 (Fig.4) may cause rectal sac distention.

8　It took four days for 20E to induce distention (Fig. 3B). Four days after injection of 20E the

9　hemolymph ecdysteroid level was 2.19 ± 0.70 μM (Fig. 4), and this concentration was not

10　sufficient to induce distention in the brain-removed pupae (Fig. 3A). These results indicate that

11　distention does not coincide with the ecdysteroid peak but requires a high level of ecdysteroid

12　concentration. In intact pupae, the ecdysteroid titer was high during P2 – P4 (Fig.4). After the

13　duration, the hemolymph ecdysteroid level decreases to a level less than 2 μM and never

14　increases again (Mizoguchi et al., 2001). The stages during P2 – P4 are therefore the stages

15　when the hemolymph ecdysteroid level is sufficiently high to induce distention. Because the

16　artificial ecdysteroid surge caused by 20E injection induced distention (Fig. 3A), we suggest

17　that distention is caused by the ecdysteroid surge during P2 – P4. Tsuchida et al. (1987)

18　suggested that the ecdysteroid peak at P2 induces follicles in developing ovarioles to enter

19　vitellogenesis in *B. mori*. In ovarian tissue, administration of 20E to an isolated pupal abdomen

20　induces morphological changes (Swevers and Iatrou, 1999). Therefore, the ecdysteroid surge

21　during P2 – P4 may have essential roles in progress of pupal-adult development in *B. mori*.

22　　　　Dedos and Fugo (1999) reported that hindgut removal from newly ecdysed pupa

23　prevented eclosion and caused a constantly high ecdysteroid level in hemolymph. However,

1   removing the hindgut from pupa 120 h after pupation (P5) did not affect eclosion. The authors

2   concluded that the rectal sac might accumulate degraded ecdysteroids from the pupal

3   hemolymph. This conclusion agrees with previous studies in *M. sexta*. The meconium (the

4   contents of the rectal sac) of *M. sexta* contained several inactivated ecdysteroids, such as

5   3-epi-20-hydroxyecdysone (Thompson et al., 1974), 20-hydroxyecdysonoic acid, and

6   3-α-epi-20, 26-dihydroxyecdysone (Warren and Gilbert, 1986). In the present results, the rectal

7   sac distended at the same stage that the ecdysteroid titer decreased (Figs. 1, 3B, and 4). It is

8   probable that the gut takes up and inactivates hemolymph ecdysteroids and that the inactivated

9   ecdysteroids accumulate in rectal sac.

10   In conclusion, rectal sac distention may be a critical step for adult development

11   because it is developmentally regulated by 20E and the sac stores meconium which contains

12   wastes. The brain–PTTH–prothoracic gland–ecdysteroid pathway thus controls the

13   developmental timing of rectal sac distention during insect metamorphosis.

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23

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23

Figure legends

2 Fig. 1. Rectal sacs distend at P4 and P5. (A) Typical rectal sacs at P3 (right) and P6 (left) are

3 shown. The rectal sac at P6 is fully distended. An arrow indicates a sac containing no meconium.

4 Scale bar = 0.5 mm. (B) Pupae were dissected at P3 – P6. The successful distention is expressed

5 as a percent ratio of the number of pupae that show distended sacs to that of total pupae. Each

6 datum is a mean of three independent experiments ± standard deviation (n = 16 - 21).

7

8 Fig. 2. Brain removal inhibits rectal sac distention. (A) The pupae had brains removed at WP

9 and P0 – P2. The brain-removed pupae were kept until P6 and dissected. The successful

10 distention is expressed as a percent ratio of the number of the pupae that show distended sacs

11 to that of total pupae. Open and closed bars indicate the occurrence in brain-removed and

12 control pupae, respectively. Each datum is a mean of three independent experiments ± standard

13 deviation (n = 15 - 19). (B) Typical rectal sacs in the P6 pupae with brains removed at WP (left)

14 and control pupae (right) are shown. An arrow indicates a sac containing no meconium. Scale

15 bar = 0.5 mm.

16

17 Fig. 3. 20E induces rectal sac distention. (A) The pupae with brains removed at WP were

18 injected with either 10 μl of 0.25 - 3.0 μg/g body weight 20E or insect Ringer's solution

19 (presented as 0) at P2 and then dissected at P8 (n = 18 – 22). (B) Four days after injection with

20 20E is sufficient time to induce distention. The pupae with brains removed at WP were injected

21 with 10 μl of 3.0 μg/g body weight 20E at P2 and then dissected at P4 – P8 (n = 16 – 18). The

22 successful distention is expressed as a percentage ratio of the number of pupae that show

23 distended sacs to the total number of pupae. Each datum is a mean of three independent

1   experiments ± standard deviation.

2

3   Fig. 4. Ecdysteroid titer from P0 to P6 in the 20E-injected pupae. Ecdysteroid was extracted

4   from the hemolymph of the pupae with brains removed at WP and injected with 20E (closed

5   circle) or insect Ringer's solution (open circle) at P2. The ecdysteroid titer was quantified by

6   radioimmunoassay. The concentration of ecdysteroid is presented as the α-ecdysone equivalent.

7   Each datum is a mean of 7 - 8 different quantifications ± standard deviation. An arrow indicates

8   the day of injection.

9

10   Fig. 5. Ecdysteroid titer in P2 pupae with brains removed at WP, P0, and P1. Ecdysteroid was

11   extracted from hemolymph of the P2 pupae with brains removed at WP, P0, and P1. As a

12   control, ecdysteroid was also extracted from intact P2 pupae. The ecdysteroid titer was

13   quantified by radioimmunoassay. The concentration of ecdysteroid is presented as the

14   α-ecdysone equivalent. Each datum is a mean of 6 – 8 different quantifications ± standard
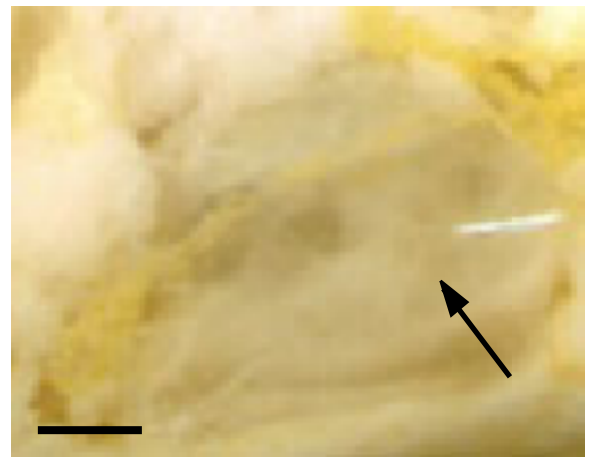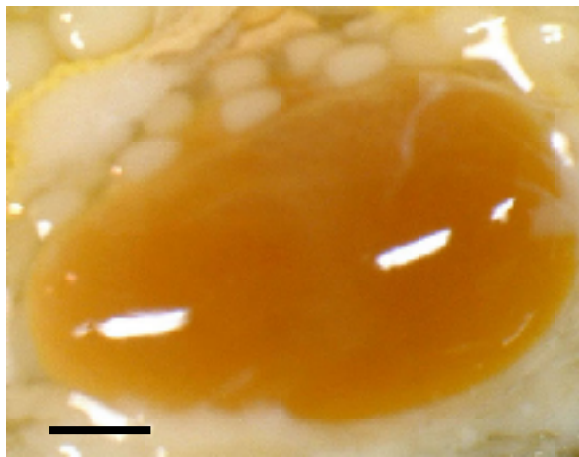
15   deviation.

Fig.1

(A)　　　　P3　　　　　　　　　　　P6

(B)

Fig.2

(A)
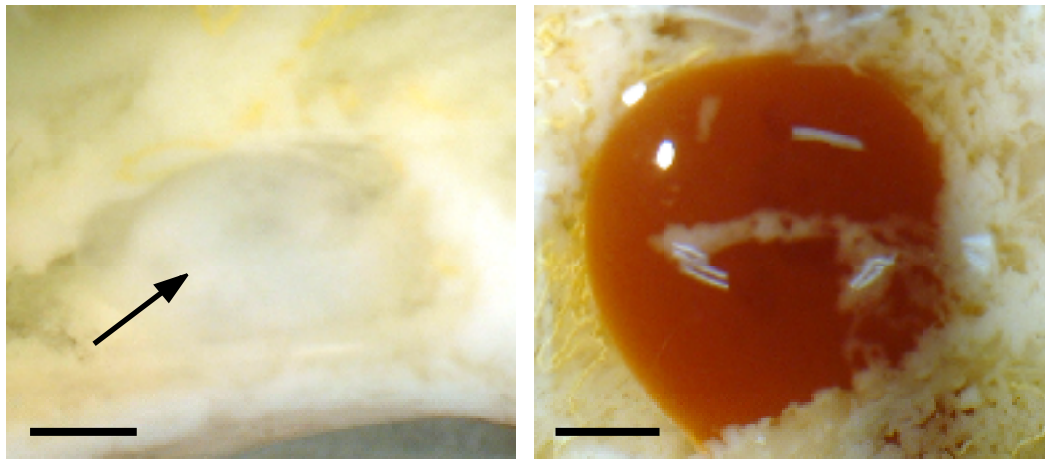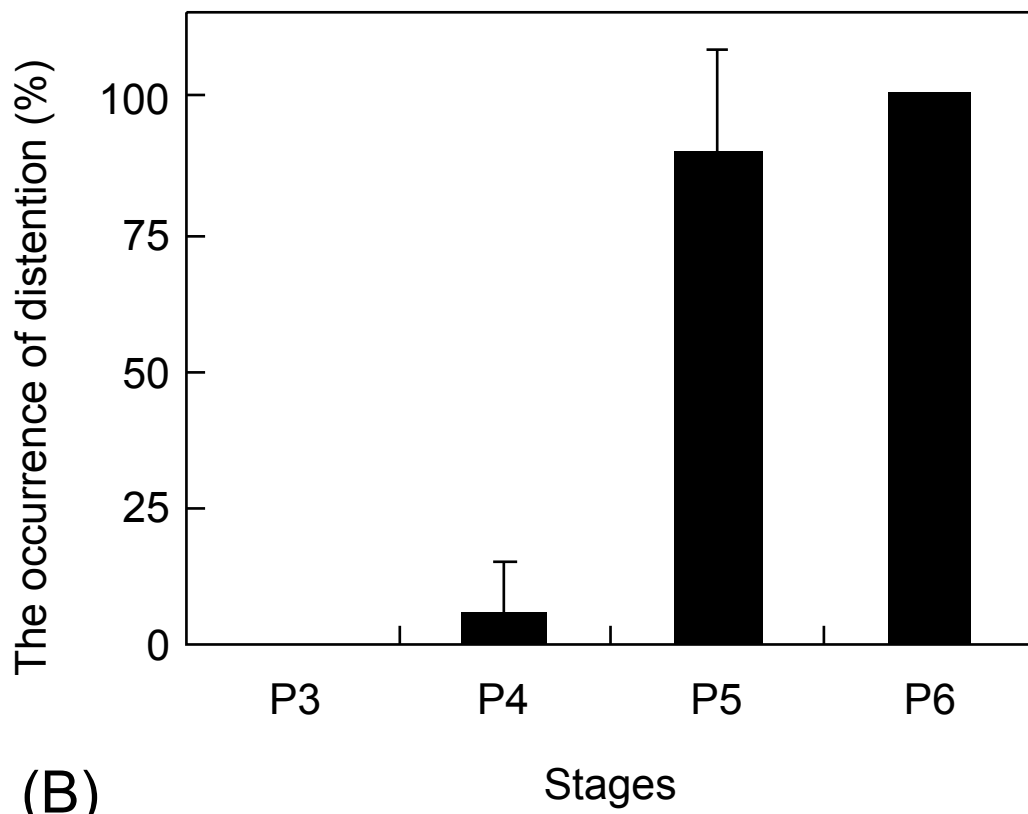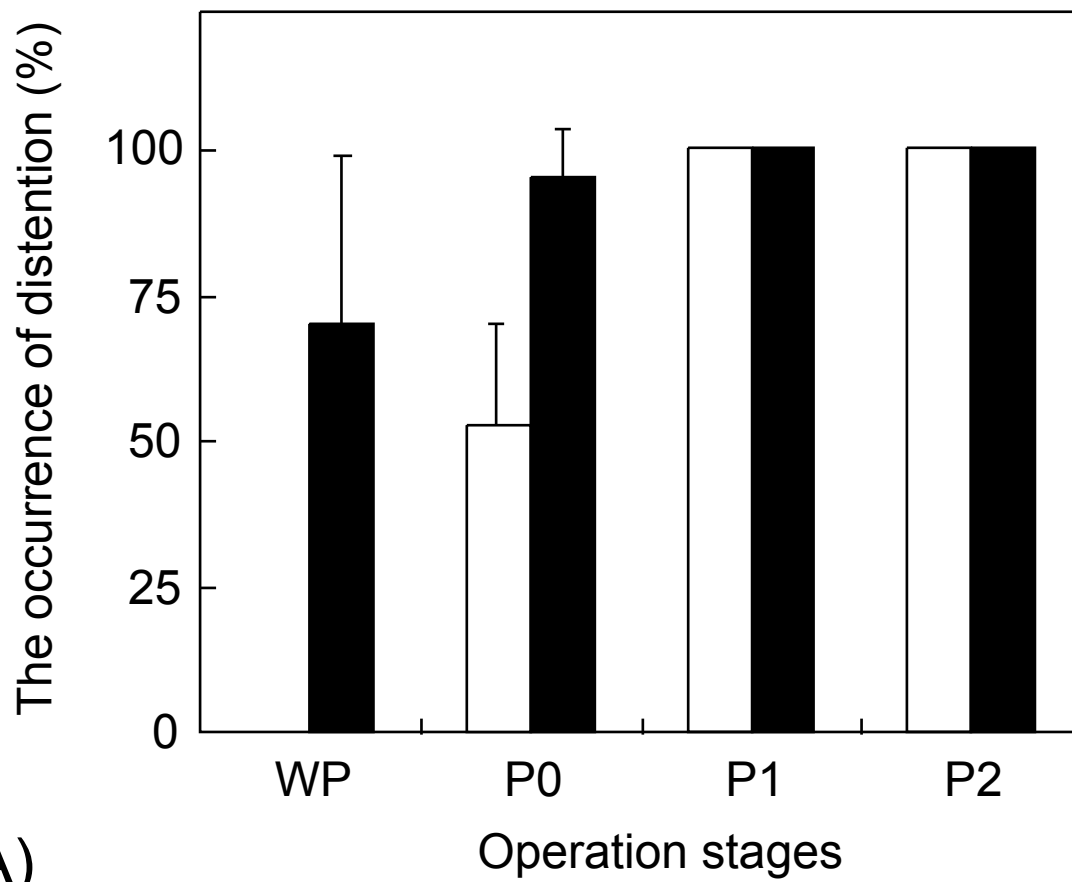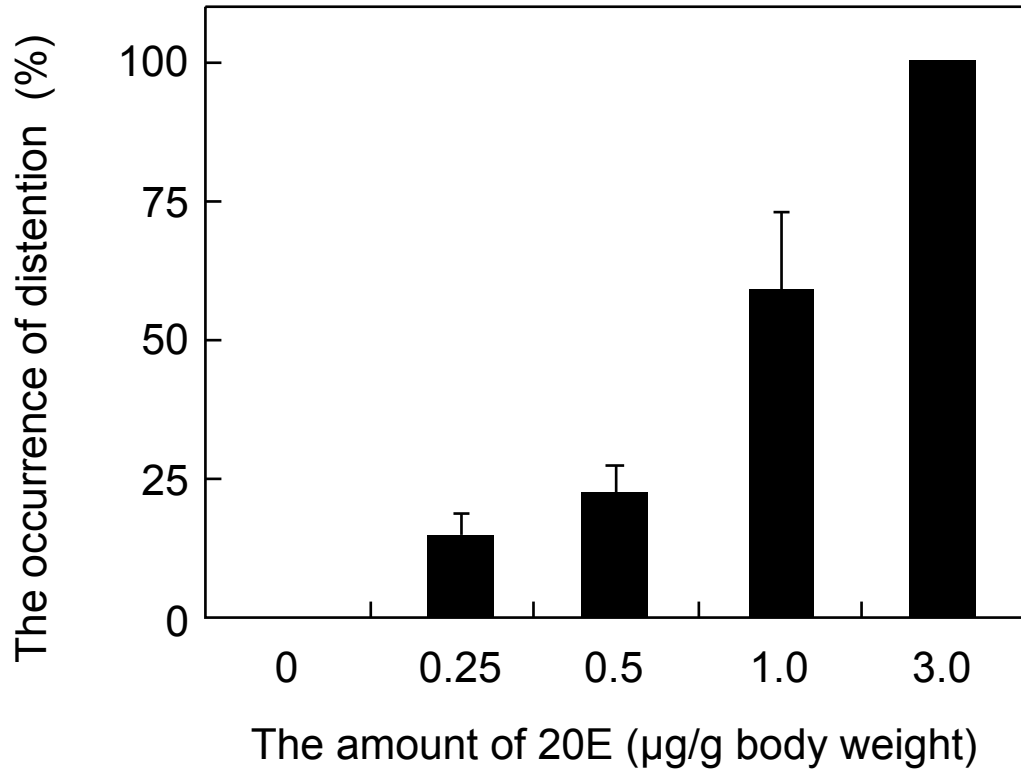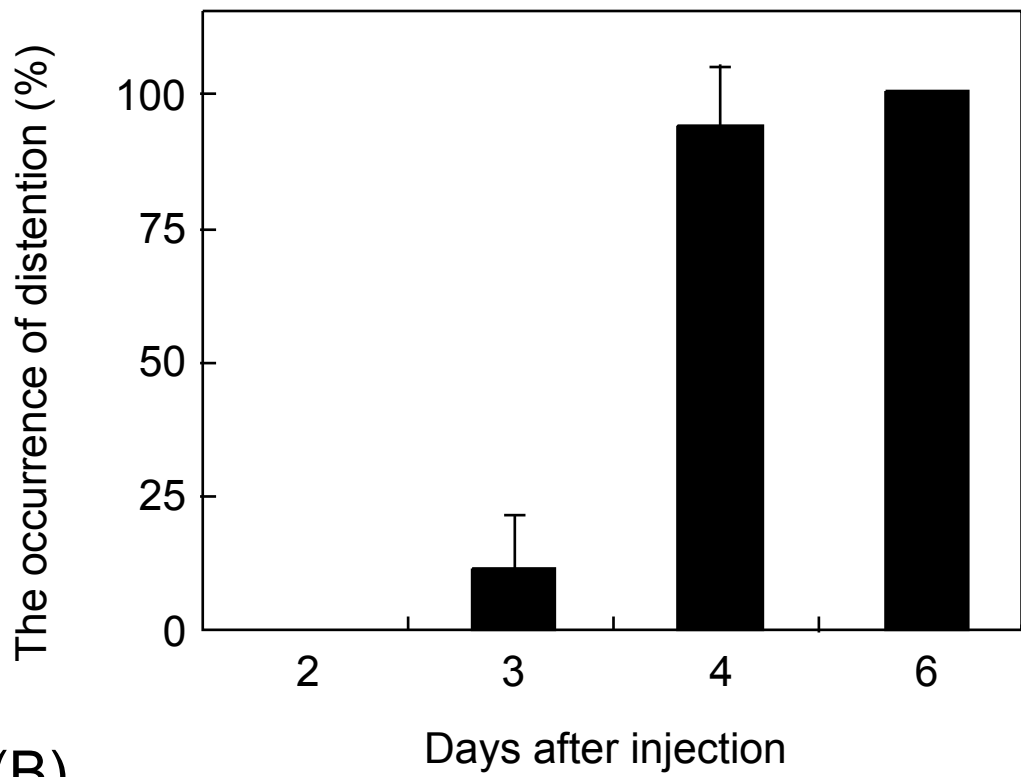
(B)   Control pupae   Brain-removed at the stage WP

Fig.3

(A)

(B)

Fig. 4

Fig. 5