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Abstract

Holometabolous insects do not excrete but store metabolic wastes during the pupal period. The waste is called meconium and is purged after adult emergence. Although the contents of meconium are well-studied, the developmental and physiological regulation of meconium accumulation is poorly understood. In *Bombyx mori*, meconium is accumulated in the rectal sac; thereby, the rectal sac distends at the late pupal stage. Here, we show that rectal sac distention occurs between 4 and 5 days after pupation. The distention is halted by brain-removal just after larval-pupal ecdysis but not by brain-removal one day after pupation. In the pupae, brain-removal just after ecdysis kept the hemolymph ecdysteroid titer low during early and mid-pupal stages. An injection of 20-hydroxyecdysone (20E) evoked the distention that was halted by brain-removal in a dose-dependent manner. Therefore, brain-removal caused the lack of ecdysteroid, and rectal sac distention did not appear in the brain-removed pupae because of the lack of ecdysteroid. We conclude that rectal sac distention is one of the developmental events regulated by 20E during the pupal period in *B. mori*.

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

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 of rectal sac distention in *B. mori* as the model of the excretory system. We show that distention occurred between 4 and 5 days after pupation. The distention was halted by brain-removal just after larval-pupal ecdysis and was evoked by 20E administration. Thus, the occurrence of rectal sac distention was developmentally controlled by 20E titer.

2. Materials and methods

2.1 Animals

B. mori (Kinshu × Showa) larvae were reared on an artificial diet (Silkmate 2M, Nihon Nosan Kougyo, Yokohama, Japan) at 25 ± 1°C under a 12 h light: 12 h dark photoperiodic regime. The day of pupation was designated as day 0 (P0). One day after pupation and 2 – 8 days after pupation were designated as stages P1 and P2 – P8, respectively. In this study, the pupae just after larval-pupal ecdysis were described as white pupae; this stage was designated as WP.

2.2 Hormones

α-ecdysone and 20E were obtained from Sigma (St Louis, MO) and dissolved in ethanol and distilled water, respectively. [^{3}H]-ecdysone (Perkin Elmer, Boston, MA) was dissolved in borate buffer (100 mM boric acid, 50 mM borax, 60 mM NaCl). 20E was diluted with insect Ringer's solution (128 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) for injections.

2.3 Operation and observations

Pupal brains were removed at WP and P0 – P2. For operational control, a hole was made in the head of each pupa and the brain was left intact. The pupae that were brain-removed at WP were injected with 10 μl of insect Ringer's solution or 20E solution. The wound made by the operation or injection was sealed with melted paraffin wax. The degree of the rectal sac distention was described as follows: no distention, in which the sac contains little or no meconium; and distention, in which the sac is filled with meconium.

2.4. Quantification of ecdysteroid titer

Hemolymph was collected from pupae by cutting the dorsal side. Ecdysteroids were extracted from hemolymph and quantified by radioimmunoassay as described previously (Sakurai et al., 1998). Anti-ecdysone antiserum H-22 was obtained from L. I. Gilbert and D. H. S. Horn and used as a capture antibody in the radioimmunoassay (Warren and Gilbert, 1986).

3. Results

3.1. Rectal sacs distended between P4 and P5

To examine when the rectal sacs distend, pupae were dissected at P3 – P6. Before P3, the rectal sacs were not observed. The sacs appeared but did not distend at P3 (Fig. 1A, left panel). A distended sac was observed in one of 17 pupae at P4 and in almost all pupae at P5 (Fig. 1B, n = 19). At P6, distended rectal sacs were observed in all pupae. These observations suggest that the rectal sac distends between P4 and P5.

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

Hemolymph titers of prothoracicotropic hormone (PTTH) (Mizoguchi et al., 2001) and bombyxin (Saegusa et al., 1992) are kept high levels during P4 and P5 and the titer of PTTH is the highest during P1 and P2 (Mizoguchi et al., 2001). We examined whether these peptides are responsible for the distention of the sacs by removing the brains from pupae at P0 – P2. The pupae were reared until P6. No distended rectal sacs appeared in the P6 pupae brain-removed at WP, while distended sacs appeared in approximately half of the P6 pupae that had their brains removed at P0 (52 ± 17%) and in all P6 pupae after brain removal at P1 or P2 (Fig. 2). The sacs were distended in most control P6 pupae operated on at WP and P0 – P2. Brains of WP pupae and P0 pupae were essential for the distention, but those of P1 or P2 were no longer essential for the distention. When the pupae were removed their brains at WP and replanted the removed brains after washing with insect Ringer's solution, the pupae showed rectal sac distention in 69% of the operated pupae (n = 13). These results indicate that a brain of WP pupa gives sufficient and necessary factor(s) for rectal sac distention.

3.3. 20E induced rectal sac distention

Figure 2 implies that the brain of a WP pupa contains an essential factor(s) for the distention. When that factor is PTTH, a lack of ecdysteroid may cause the failure of the distention in P6 pupa that had their brains removed at WP. We injected 20E (0.25 - 3.0 μg/g body weight) to P2 pupae brain-removed at WP. The injected pupae were reared until P8. As shown in Figure 3, 3.0 μg/g body weight 20E induced distention in all injected pupae. Over the range from 0.25 to 3.0 μg/g body weight, 20E induced distention in a dose-dependent manner. Insect Ringer's solution did not induce the distention. This result shows that distention is induced by 20E, indicating that a lack of 20E causes the failure of distention in pupae with brains removed at WP.

We examined contributions of juvenile hormone in rectal sac distention by allatectomy in fourth instar larvae. Distended rectal sacs appeared in 93% of allatectomized precocious pupae on day 6 (n=14). The source of juvenile hormone, the corpora allata, was therefore not essential for the distention.

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

We examined what time period is sufficient to induce the distention caused by 20E. We injected 20E to the brain-removed pupae at P2 and dissected the pupae at P4 – P8. Distended sacs appeared in most pupae at P6 and in all pupae at P8 (Fig. 3B). They did not appear at P4 and appeared in a few pupae at P5. The appearance of the distention after P6 indicates that the rectal sac distention is induced four days after 20E-injection.

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

Figure 3 shows that the rectal sacs were distended at P6 of 20E-injected pupae. We determined the ecdysteroid titer of 20E-injected pupae from P0 to P6. The titers of 20E-injected pupae were 4.36 ± 0.37 μM and 4.44 ± 0.72 μM at 1 and 2 days after injection, respectively (Fig. 4). Then, it decreased sharply to reach the control level. The titer of ecdysteroid decreased at P5 in the 20E-injected pupae. In the control experiment, the ecdysteroid titer was kept at a constant level (1.05 – 1.78 μM, n = 7 - 8).

3.6 Ecdysteroid elevation was inhibited in a stage specific manner

Brain-removal at WP inhibited rectal sac distention completely, but brain-removal at P1 did not (Fig. 2). We examined whether the difference of inhibition by brain-removal was caused by ecdysteroid. Ecdysteroid was extracted from the P2 pupae with brains removed at

WP, P0, and P1, and then its amount was measured by radioimmunoassay. The levels of ecdysteroid were 4.20 ± 1.09 μM and 5.62 ± 0.61 μM in the pupae operated at P0 and P1, respectively (Fig. 5). By contrast, the level was 1.05 ± 0.27 μM in the pupae operated at WP. Thus, ecdysteroid elevation was inhibited by brain-removal at WP.

4. Discussion

Here, we show that rectal sac distention occurs during P4 – P5. The distention is halted by brain-removal from WP pupae, and distention resumes after 20E injection. The resumption of distention after 20E injection indicates that a lack of ecdysteroid prevents the sac from distending. Ecdysteroid production in the prothoracic gland, an ecdysteroidogenic organ, is activated by PTTH secreted from the brain (Kawakami et al., 1990). Brain-removal causes a lack of PTTH and, thereby, a decrease in the ecdysteroid level. Thus, the brain is essential for distention by regulating ecdysteroidogenesis.

The ecdysteroid titer was high during P2 – P4 (Fig. 4) and declines sharply to an undetectable level at the time of eclosion (Mizoguchi et al., 2001). In the pupae that had brains removed and had been injected with insect Ringer's solution, the hemolymph ecdysteroid level was kept at concentrations ranging from 1.05 ± 0.27 to 1.78 ± 0.39 μM during P0 – P6 (Fig. 4). The amount of PTTH was expressed as *Bombyx* unit as in a previous study (Ishizaki et al., 1983). A *Bombyx* unit is defined as the minimum amount of PTTH necessary to induce adult development in more than half of the brain-removed pupae. A *Bombyx* unit of PTTH is equivalent to 110 pg (Kataoka et al., 1987). The PTTH concentration of the newly ecdysed pupa is approximately 100 pg/ml (Mizoguchi et al., 2001), and the newly ecdysed pupae therefore contain enough PTTH in their hemolymph to initiate adult development. However, the brain-removed pupae never initiate adult development in *B. mori* (Kobayashi and Kimura, 1958). When the brain-removed pupae were injected with brain extract, they initiate adult development. Figure 5 shows that brain-removal at WP significantly inhibited ecdysteroid elevation and brain-removal at P0 and P1 did not. Therefore, the pupae brain-removed at WP did not contain enough PTTH to activate the prothoracic glands, and the failure of the rectal sac to distend may be due to the lack of PTTH.

The higher ecdysteroid level during P2 – P4 (Fig.4) may cause rectal sac distention. It took four days for 20E to induce distention (Fig. 3B). Four days after injection of 20E the hemolymph ecdysteroid level was 2.19 ± 0.70 μM (Fig. 4), and this concentration was not sufficient to induce distention in the brain-removed pupae (Fig. 3A). These results indicate that distention does not coincide with the ecdysteroid peak but requires a high level of ecdysteroid concentration. In intact pupae, the ecdysteroid titer was high during P2 – P4 (Fig.4). After the duration, the hemolymph ecdysteroid level decreases to a level less than 2 μM and never increases again (Mizoguchi et al., 2001). The stages during P2 – P4 are therefore the stages when the hemolymph ecdysteroid level is sufficiently high to induce distention. Because the artificial ecdysteroid surge caused by 20E injection induced distention (Fig. 3A), we suggest that distention is caused by the ecdysteroid surge during P2 – P4. Tsuchida et al. (1987) suggested that the ecdysteroid peak at P2 induces follicles in developing ovarioles to enter vitellogenesis in *B. mori*. In ovarian tissue, administration of 20E to an isolated pupal abdomen induces morphological changes (Swevers and Iatrou, 1999). Therefore, the ecdysteroid surge during P2 – P4 may have essential roles in progress of pupal-adult development in *B. mori*.

Dedos and Fugo (1999) reported that hindgut removal from newly ecdysed pupa prevented eclosion and caused a constantly high ecdysteroid level in hemolymph. However, removing the hindgut from pupa 120 h after pupation (P5) did not affect eclosion. The authors concluded that the rectal sac might accumulate degraded ecdysteroids from the pupal hemolymph. This conclusion agrees with previous studies in *M. sexta*. The meconium (the contents of the rectal sac) of *M. sexta* contained several inactivated 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). In the present results, the rectal sac distended at the same stage that the ecdysteroid titer decreased (Figs. 1, 3B, and 4). It is probable that the gut takes up and inactivates hemolymph ecdysteroids and that the inactivated ecdysteroids accumulate in rectal sac.

In conclusion, rectal sac distention may be a critical step for adult development because it is developmentally regulated by 20E and the sac stores meconium which contains wastes. The brain–PTTH–prothoracic gland–ecdysteroid pathway thus controls the developmental timing of rectal sac distention during insect metamorphosis.

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References

Brown, W. A., 1938. The nitrogen metabolism of an insect (*Lucilia sericata* MG.) I. Uric acid, allantoin and uricase. Biochemistry Journal 32, 895-902.

Dedos, S. G., Fugo, H., 1999. Disturbance of adult eclosion by fenoxycarb in the silkworm, *Bombyx mori*. Journal of Insect Physiology 45, 257-264.

Ishizaki, H., Suzuki, A., Moriya, I., Mizoguchi, A., Fujishita, M., O'oka, H., Kataoka, H., Isogai, A., Nagasawa, H., Suzuki, A., 1983. Prothoracicotropic hormone bioassay: pupal-adult *Bombyx* assay. Development Growth & Differentiation 25, 585-592.

Judy, K. J., Gilbert, L. I., 1970a. Histology of the alimentary canal during the metamorphosis of *Hyalophora cecropia* (L.). The Journal of Morphology 131, 277-300.

Judy, K. J., Gilbert, L. I., 1970b. Effects of juvenile hormone and molting hormone on rectal pad development in *Hyalophora cecropia* (L.). The Journal of Morphology 131, 301-314.

Kataoka, H., Nagasawa, H., Isogai, A., Tamura, S., Mizoguchi, A., Fujiwara, Y., Suzuki, C., Ishizaki, H., Suzuki, A., 1987. Isolation and partial characterization of a prothoracicotropic hormone of silkworm, *Bombyx mori*. Agricultural and Biological Chemistry 51, 1067-1076.

Kawakami, A., Kataoka, H., Oka, T., Mizoguchi, A., Kimura-Kawakami, M., Adachi, T., Iwami, M., Nagasawa, H., Suzuki, A., Ishizaki, H., 1990. Molecular cloning of the *Bombyx mori* prothoracicotropic hormone. Science 247, 1333-1335.

Kiguchi, K., Agui, N., Kawasaki, H., Kobayashi, K., 1985. Developmental time-table for the last larval and pharate pupal stages in the silkworm, *Bombyx mori*, with special reference to the correlation between the developmental events and hemolymph ecdysteroid levels. Bulletin of the Sericultural Experimental Station 30, 83-100.

Kobayashi, M., Kimura, J., 1958. The 'brain' hormone in the silkworm, *Bombyx mori* L. Nature 181, 1217.

Levenbook, L., Hutchins, R. F. N., Bauer A. C., 1971. Uric acid and basic amino acids during metamorphosis of the tobacco hornworm, *Menduca sexta*, with special reference to the meconium. Journal of Insect Physiology 17, 1321-1331.

Mizoguchi, A., Ohashi, Y., Hosoda, K., Ishibashi, J., Kataoka, H., 2001. Developmental profile of the changes in the prothoracicotropic hormone titer in hemolymph of the silkworm *Bombyx mori*: correlation with ecdysteroid secretion. Insect Biochemistry and Molecular Biology 31, 349-358.

Nagata, H., Ohtaki, T., Sakurai, S., 1987. Role of low ecdysteroid titre in acquisition of competence for pupal transformation in *Bombyx mori*. Journal of Insect Physiology 33, 657-662.

Saegusa, H., Mizoguchi, A., Kitahora, H., Nagasawa, H., Suzuki, A., Ishizaki, H., 1992. Changes in the titer of bombyxin-immunoreactive material in hemolymph during the postembryonic development of the silkmoth *Bombyx mori*. Development Growth & Differentiation 34, 595-605.

Sakurai, S., Kaya, M., Satake, S., 1998. Hemolymph ecdysteroid titer and ecdysteroid-dependent developmental events in the last-larval stadium of the silkworm, Bombyx mori: role of low ecdysteroid titer in larval-pupal metamorphosis and a reappraisal of the head critical period. Journal of Insect Physiology 44, 867-881.

Swevers, L., Iatrou, K., 1999. The ecdysone agonist tebufenozide (RH-5992) blocks the progression into the ecdysteroid-induced regulatory cascade and arrests silkmoth oogenesis at mid-vitellogenesis. Insect Biochemistry and Molecular Biology 29, 955-963.

Thompson, M. J., Kalpains, J. N., Robbins, W. E., Dutky, S. R., Nigg, H. N., 1974. 3-Epi-20-hydroxyecdysone from meconium of tabacco hornworm. Steroids 24, 359-366.

Tsuchida, K., Nagata, M., Suzuki, A., 1987. Hormonal control of ovarian development in the silkworm, *Bombyx mori*. Archives of Insect Biochemistry and Physiology 5, 167-177.

Warren, J. T. and Gilbert, L. I., 1986. Ecdysone metabolism and distribution during the pupal-adult development of *Manduca sexta*. Insect Biochemistry 16, 65-82.

Figure legends

Fig. 1. Rectal sacs distend at P4 and P5. (A) Typical rectal sacs at P3 (right) and P6 (left) are shown. The rectal sac at P6 is fully distended. An arrow indicates a sac containing no meconium. Scale bar = 0.5 mm. (B) Pupae were dissected at P3 – P6. The successful distention is expressed as a percent ratio of the number of pupae that show distended sacs to that of total pupae. Each datum is a mean of three independent experiments ± standard deviation (n = 16 - 21).

Fig. 2. Brain removal inhibits rectal sac distention. (A) The pupae had brains removed at WP and P0 – P2. The brain-removed pupae were kept until P6 and dissected. The successful distention is expressed as a percent ratio of the number of the pupae that show distended sacs to that of total pupae. Open and closed bars indicate the occurrence in brain-removed and control pupae, respectively. Each datum is a mean of three independent experiments ± standard deviation (n = 15 - 19). (B) Typical rectal sacs in the P6 pupae with brains removed at WP (left) and control pupae (right) are shown. An arrow indicates a sac containing no meconium. Scale bar = 0.5 mm.

Fig. 3. 20E induces rectal sac distention. (A) The pupae with brains removed at WP were injected with either 10 μl of 0.25 - 3.0 μg/g body weight 20E or insect Ringer's solution (presented as 0) at P2 and then dissected at P8 (n = 18 – 22). (B) Four days after injection with 20E is sufficient time to induce distention. The pupae with brains removed at WP were injected with 10 μl of 3.0 μg/g body weight 20E at P2 and then dissected at P4 – P8 (n = 16 – 18). The successful distention is expressed as a percentage ratio of the number of pupae that show distended sacs to the total number of pupae. Each datum is a mean of three independent

experiments ± standard deviation.

Fig. 4. Ecdysteroid titer from P0 to P6 in the 20E-injected pupae. Ecdysteroid was extracted from the hemolymph of the pupae with brains removed at WP and injected with 20E (closed circle) or insect Ringer's solution (open circle) at P2. The ecdysteroid titer was quantified by radioimmunoassay. The concentration of ecdysteroid is presented as the α-ecdysone equivalent. Each datum is a mean of 7 - 8 different quantifications ± standard deviation. An arrow indicates the day of injection.

Fig. 5. Ecdysteroid titer in P2 pupae with brains removed at WP, P0, and P1. Ecdysteroid was extracted from hemolymph of the P2 pupae with brains removed at WP, P0, and P1. As a control, ecdysteroid was also extracted from intact P2 pupae. The ecdysteroid titer was quantified by radioimmunoassay. The concentration of ecdysteroid is presented as the α-ecdysone equivalent. Each datum is a mean of 6 – 8 different quantifications ± standard deviation.

Fig.1

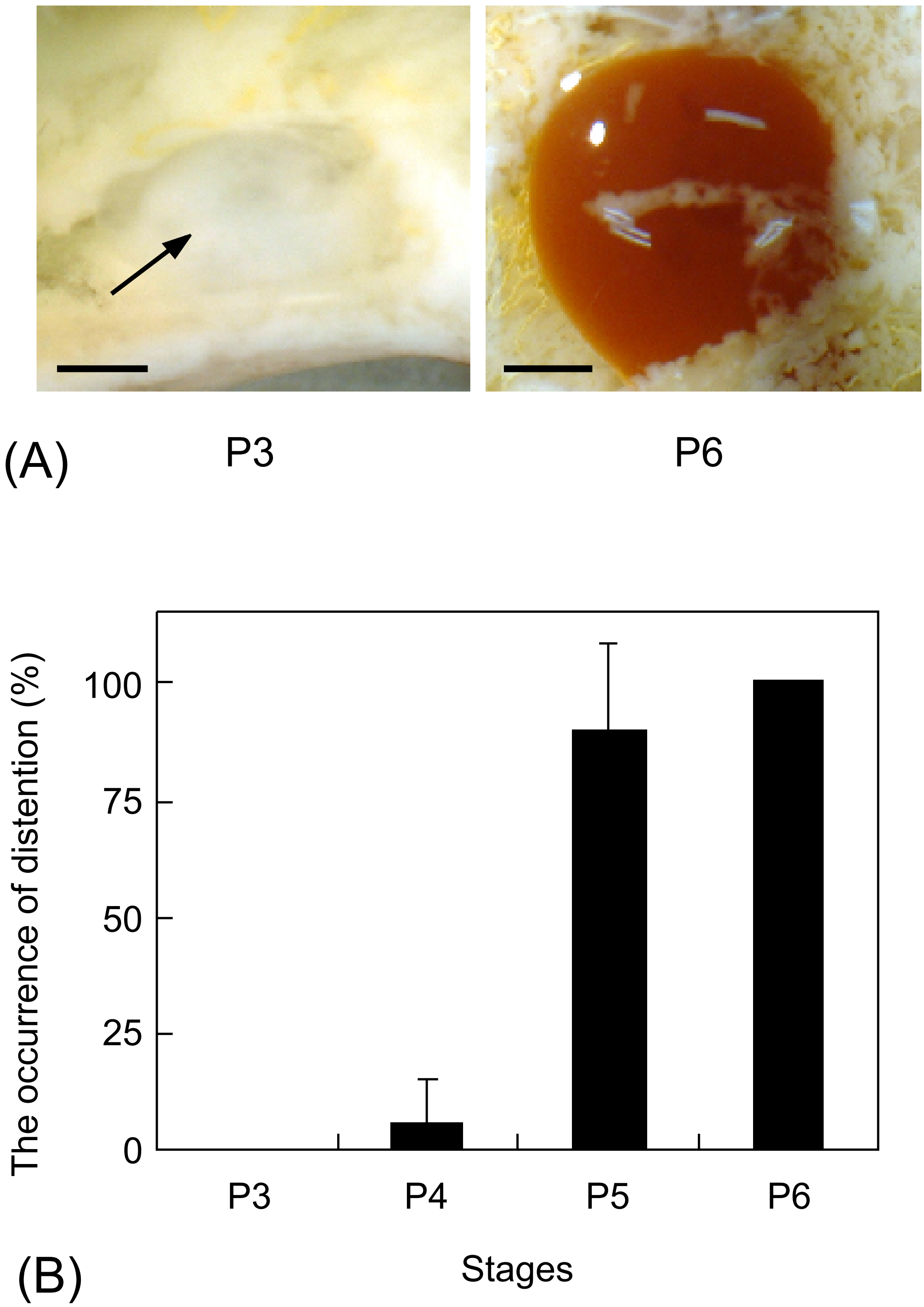
(A)
P3
P6
(B)
The occurrence of distention (%)
100
75
50
25
0
P3
P4
P5
P6
Stages

Fig.2

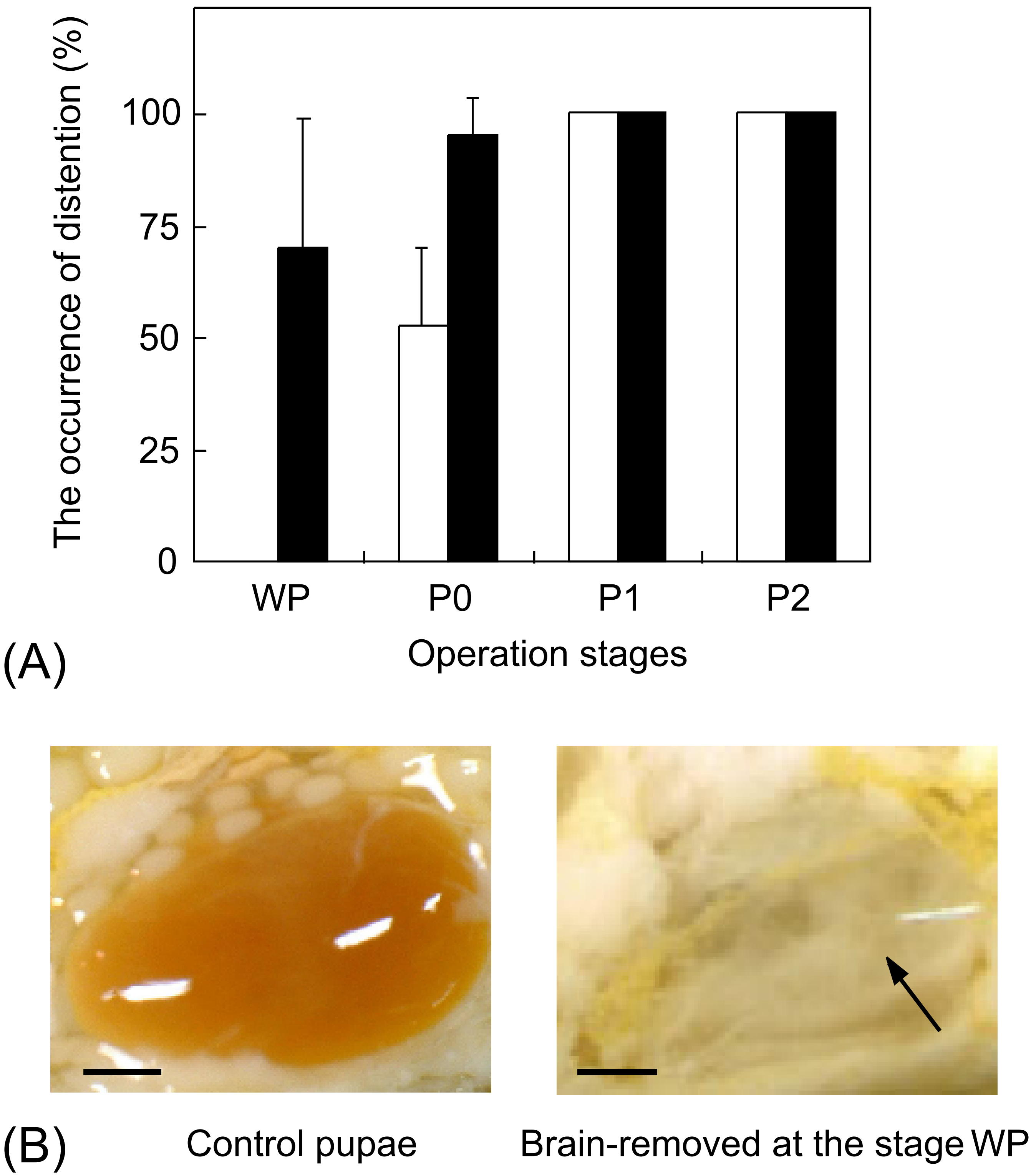
The occurrence of distention (%)
0
25
50
75
100
WP
P0
P1
P2
(A)
Operation stages
(B)
Control pupae
Brain-removed at the stage WP

Fig.3

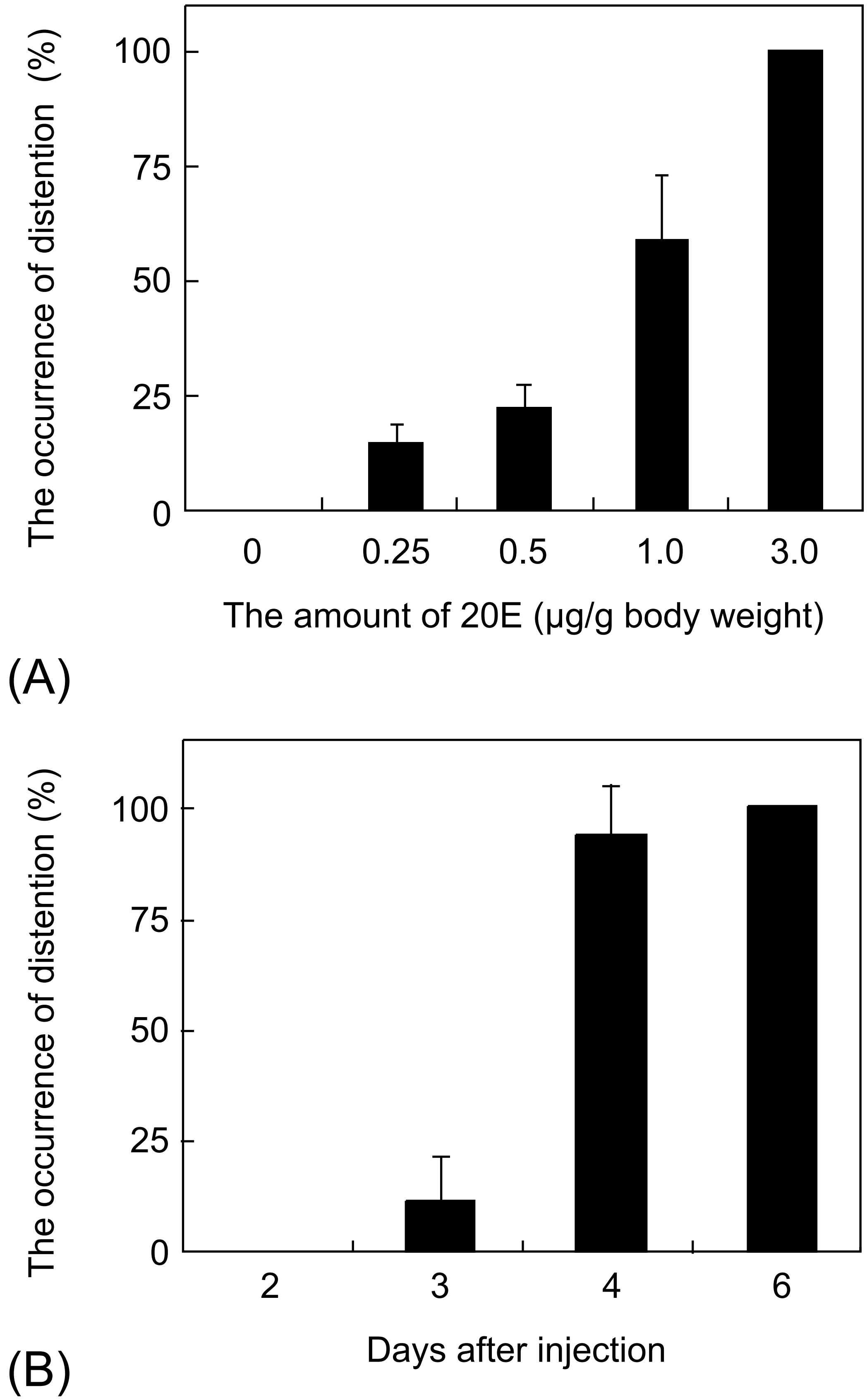
The occurrence of distention (%)
100
75
50
25
0
0
0.25
0.5
1.0
3.0
The amount of 20E (µg/g body weight)
(A)
The occurrence of distention (%)
100
75
50
25
0
2
3
4
6
Days after injection
(B)

Fig. 4

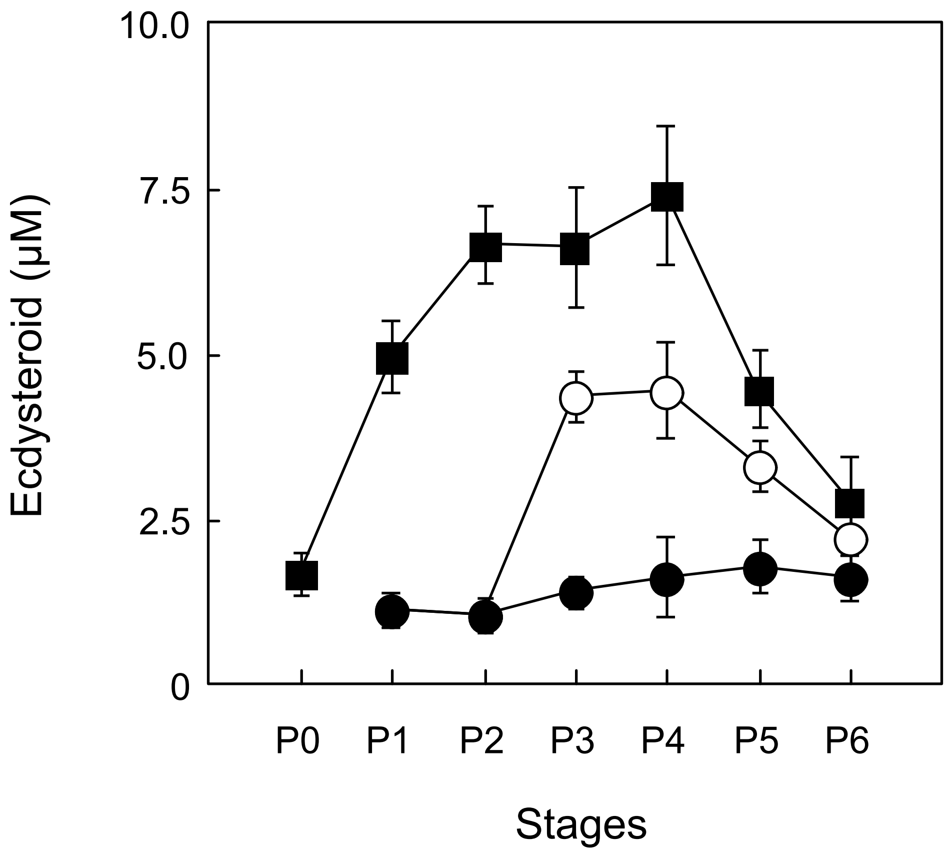

Fig. 5