Juvenile hormone delays the initiation of rectal sac distention by disrupting ecdysteroid action in the silkworm, Bombyx mori

Juvenile hormone delays the initiation of rectal sac distention by disrupting ecdysteroid action in the silkworm, *Bombyx mori*

Takumi Suzuki, Sho Sakurai, and Masafumi Iwami*

Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

* Corresponding author: Masafumi Iwami, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kakuma-machi, Kanazawa 920-1192, Japan

Tel: +81 76 264 6251

Fax: +81 76 264 6215

E-mail: masafumi@kenroku.kanazawa-u.ac.jp

Abstract

Holometabolous insects develop without feeding and excreting during the pupal period and thus require repository organs for metabolic waste, or meconium. The rectal sac is an organ for storing meconium during pupal-adult development of holometabolous insects. Although the rectal sac has an essential function, hormonal and developmental regulation of waste-accumulation and the consequences of rectal sac distention are still unknown. In the silkworm, *Bombyx mori*, the rectal sac distends with meconium in the middle pupal period under the regulation of ecdysteroid. Here, we show that juvenile hormone analog (JHA) delayed rectal sac distention and disturbed adult emergence. Distention was not restored completely by an injection of 20-hydroxyecdysone (20E) into pupae applied with JHA, suggesting that JHA suppresses 20E action and delays the timing of ecdysteroid elevation. Thus the "status quo" action of JHA may function in two different ways during pupal-adult development.

Keywords: excretory system, ecdysone, juvenile hormone, metamorphosis, adult emergence

1. Introduction

During the pupal period, insects do not feed or excrete metabolic waste. The excretory system of insects consists of Malphigian tubules, an alimentary canal, and a rectal sac. Malphigian tubules generate primary urine and play an osmoregulatory role, and the alimentary canal has both a digestive and an excretory function. The rectal sac is one of the essential organs for storing metabolic waste, or meconium. Meconium is kept in the sac throughout the pupal period and discharged after adult emergence. Inactivated ecdysteroids, such as 3-epi-20-hydroxyecdysone and 20-hydroxyecdysonoic acid, were detected in the meconium, indicating that the sac could be responsible for the clearance of inactivated ecdysteroids from the hemolymph [1]. The rectal sac thus plays an important role in successful adult development. In a previous study, we found that rectal sacs of the silkworm, *Bombyx mori*, were distended with meconium in the middle of pupal-adult development; the distention was induced by the administration of 20-hydroxyecdysone (20E) in a dose-dependent manner [2]. The distention was halted when hemolymph ecdysteroid titer was lowered by brain removal [2] and the pupae without the sac failed to eclose [3].

Juvenile hormone (JH) regulates insect development as “status quo hormone” by directing or antagonizing ecdysteroid action [4]. JH effects were studied in the alimentary canal

and in the Malpighian tubules. In the giant silk moth, *Hyalophora cecropia*, the alimentary canal undergoes mitosis and histological change during pupal-adult development, but these developments are inhibited by injection with a high dose of JH [5]. In the tobacco budworm, *Heliothis virescens*, the larval midgut undergoes a programmed cell death during metamorphosis; this process can be blocked by application of JH analog (JHA), methoprene [6]. In the pupae of skipper butterflies, *Calpodes ethlius*, 20E causes Malpighian tubules to undergo morphological remodeling and halt fluid secretion. When *C. ethlius* was treated with JH, Malpighian tubules were not remodeled and continued to secrete fluids [7]. In *B. mori*, fenoxycarb, another JHA, blocks rectal sac distention [3]. Thus, the actions of JH in the excretory system are important but not well understood.

JH is regarded as an insect growth regulator and its action has been studied in the agricultural field for pest managing and crop protection. So far, most of the study had been focused on the potency of JH as a larval growth regulator. However, its effects were not studied well against pupal and adult stages. JH effect has to be studied as the growth regulatory potency to pupae and adult because adults of some species cause high loss of crops.

In the present study, we examine the pharmacological and physiological potency of

JHAs in the distention of the rectal sac as a model. We show that the timings of distention and ecdysteroid elevation are delayed in pupae applied with methoprene. Our results suggest that the "status quo" action of JH [4] occurs by modifying actions of 20E and causing changes in the ecdysteroid titer. We also discuss the effect of JHA on pupa of lepidopteran species as an insect growth regulator.

2. Materials and methods

2.1 Animals

B. mori (Kinshu × Showa) larvae were reared on an artificial diet (Silkmate 2M, Nihon Nosan Kougyo, Yokohama) at 25 ± 1˚C under a 12 h light: 12 h dark photoperiod. The day of pupation was designated as day 0 (P0). One day and 2 – 9 days after pupation were designated as stages P1 and P2 – P9, respectively.

2.2 Hormones and observation

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, 5 mM borax, 60 mM NaCl), and 20E was diluted with insect Ringer's solution (128 mM NaCl, 4.7 mM KCl, 1.9 mM $CaCl_2$) for injections. S-methoprene (SDS Biotech, Tokyo) and fenoxycarb (Wako Pure Chemical Industries, Osaka) were dissolved in acetone, and 10 μl of each chemical was applied to the dorsal surface of individual pupae at various dosages per gram body weight during P0 – P2. The applied dosage of the chemicals was mentioned in each experiment; otherwise, 5 mg/g was applied. The degree of rectal sac distention was as described previously [2].

2.3. Quantification of ecdysteroid titer

The hemolymphs samples were collected from pupae by cutting the dorsal side. Ecdysteroids were extracted from the hemolymphs and quantified by radioimmunoassay as described previously [8]. 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 [1].

3. Results

3.1 JHA suppressed rectal sac distention at P6

We applied various doses of methoprene and fenoxycarb to P0 pupae and dissected the pupae at P6. When 1–1000 μg/g of methoprene or 1–100 μg/g of fenoxycarb was applied to pupae, rectal sac distention was observed in over 80% of the pupae (Table 1). Distention appeared in 1 of 18 and 3 of 7 pupae applied with 5 mg/g of methoprene and 1 mg/g of fenoxycarb, respectively. Distention was observed in most of the pupae applied with acetone as a control. These results indicate that a high dose of JHA application suppressed rectal sac distention at P6.

3.2 JHA suppressed rectal sac distention in a stage-specific manner

We examined the time at which rectal sac distention was suppressed by JHA. Methoprene was applied to pupae at one stage within stages P0 – P2, and the pupae were dissected at P6. The distended sacs appeared in less than 20% of the P6 pupae that had been applied methoprene at P0 and approximately 40% of the P6 pupae that received methoprene at P1 (Fig. 1). When methoprene was applied to pupae at P2, the distended sacs appeared in all pupae. These results indicate that methoprene application at P0 and P1 suppressed rectal sac distention, but application at P2 did not.

3.3 JHA altered hemolymph ecdysteroid titer

We reported previously that 20E induces rectal sac distention in a dose-dependent manner [2]; distention did not occur in the presence of methoprene (Fig. 1). This result raises the possibility that methoprene lowers the ecdysteroid level in the hemolymph and that the level of ecdysteroid may not be high enough for distention. We therefore quantified the hemolymph ecdysteroid titer from P0 – P6 in the pupae applied with methoprene. The hemolymph was extracted from P1 – P6 pupae applied with methoprene at P0 and from pupae applied with acetone at P0 as controls. The hemolymph of P0 pupae was also extracted before application to quantify an initial ecdysteroid level. The hemolymph ecdysteroid titer in control pupae peaked at P2 and then gradually decreased to a level of less than 2 μM at P6 (Fig. 2). In pupae applied with methoprene, the ecdysteroid titer gradually increased until P6, reaching a level of more than 10 μM. Methoprene therefore did not inhibit ecdysteroid elevation completely, but did cause a delayed increase in ecdysteroid titer.

3.4 JHA delayed timing of rectal sac distention

Figure 2 shows that methoprene application resulted in a delay in the increase of hemolymph ecdysteroid titer. In a previous study, we concluded that an ecdysteroid surge occurring at P2 – P4 was essential for rectal sac distention [2]. In pupae applied with methoprene, the ecdysteroid titer was lower than that of control pupae at P2, but was elevated significantly to reach higher than that of control at a later stage (Fig. 2). When the ecdysteroid titer was high enough to induce rectal sac distention, the distention occurred at a later stage in the pupae applied with methoprene. We therefore examined the occurrence of successful distention during P6 – P9 in pupae applied with methoprene. Distention occurred in 19 ± 17% of pupae at P6 and in more than half of the pupae at P7 (58 ± 22%, $p = 0.044$), P8 (69 ± 11%, $p = 0.0028$), and P9 (53 ± 14%, $p = 0.039$) (Fig. 3). Between P7 and P9, there were no significant differences in the ratio of successful distention. Methoprene thus did not inhibit the timing of distention, but rather delayed it.

3.5 JHA inhibited adult emergence in a stage-specific manner

Dedos and Fugo (1999) reported that fenoxycarb injections inhibit eclosion behavior in *B. mori*. They suggest that the disturbance of adult eclosion is due in part to rectal sac

distention failure, since fenoxycarb treatment inhibited distention, and ablation of the rectal sac resulted in disturbance of eclosion. However, Figure 3 shows methoprene application did not inhibit distention but delayed the timing of the distention. We therefore examined the effect of methoprene on eclosion. The P0 and P2 pupae were applied methoprene or acetone as a control and kept at 25˚C. All of the pupae applied with acetone eclosed 10 – 13 days after application (Table 2). When pupae were applied methoprene at P0, they did not show any sign of eclosion, even 21 days after application. When pupae were applied methoprene at P2, 60% of the pupae eclosed (Table 2). Thus, methoprene application inhibited adult emergence in a stage-specific manner.

3.6 JHA interrupted 20E-induced rectal sac distention

The above results indicate that JHA altered the ecdysteroid titer. We further examined whether artificial ecdysteroid elevation restores distention in pupae applied with methoprene. The pupae were applied methoprene at P0, injected with 3.0 μg/g 20E at P1, and dissected at a stage between P4 – P6. Successful distention occurred in 17 ± 19% of the pupae applied with methoprene and 90 ± 3% of the control pupae at P4. At P5, distention occurred in 44 ± 15% of

the pupae applied with methoprene and 93 ± 13% of the control pupae (Fig. 4). In contrast, the ratio of successful distention decreased at P6 in the pupae applied with methoprene (17 ± 10%). In several P6 pupae, meconium, leaked meconium, and murky hemolymph with meconium were observed in the hindgut, a layer between the newly formed and pupal cuticles, and the abdominal region, respectively. Successful distention occurred in all of the control pupae at P6. There was no statistically meaningful difference between the ratio of successful distention in P5 pupae applied with methoprene and that of P6 pupae applied with methoprene (Student's t-test). Injection of 20E therefore did not restore distention in the pupae that received an application of methoprene.

The result implies that JHA interrupted rectal sac distention by altering ecdysteroid titer and suppressing its action. We analyzed JHA effects on brain-removed pupae to confirm that JHA suppressed 20E action. We previously reported that rectal sac distention was halted by brain-removal just after pupal ecdysis and restored by 20E in a dose dependent manner [2]. Ecdysteroid level did not increase at least until P6 in brain-removed pupae. Thus, interference of endogenous ecdysteoid was negligible in the brain-removed pupae to allow assessing directly the action of injected 20E. Therefore, pupae were brain-removed just after ecdysis, applied with

methoprene or acetone as control at P1, and injected with 20E at P2. The pupae were then dissected at P8. In both 1.0 and 3.0 μg of 20E injected pupae, JHA reduced the number of pupae showing successful distention (Fig. 5). Acetone application did not interrupt induction of successful distention by 20E. Thus, JHA interrupted 20E action itself in rectal sac distention.

4. Discussion

JH was studied in many insect species as an insect growth regulator. Most studies have been focused on its effect on larval growth regulation. In the present study, we examined pharmacological and physiological effects of JHA on the pupae of *B. mori*. We demonstrated that methoprene and fenoxycarb delay the timing of rectal sac distention. The delay of distention was caused by methoprene (5 mg/g) applied at P0 – P1, but no delayed distention was observed when P2 pupae were applied the same dose of methoprene (Fig. 1). Although 5 mg/g was higher than a physiological dose, the delay was probably caused by JH activity of methoprene because its application delayed the timing of rectal sac distention in a stage-specific manner (Table 2). We therefore determined that the suppression was caused by JH activity of methoprene. Although Dedos and Fugo (1999) reported that an injection of 1 μg of fenoxycarb

was sufficient for the suppression of rectal sac distention, we did not obtain the same result. An application of 1 mg/g fenoxycarb suppressed distention in half of the treated pupae (Table 1). These conflicting results may be caused by the permeability of fenoxycarb against the pupal cuticle. Applied fenoxycarb might barely enter the pupal body because it penetrates the cuticle poorly. In the larvae of the tobacco hornworm, *Manduca sexta*, even an application of 50 μg of JH-I resulted in a JH titer increase to approximately 1 μM [9]. Thus, the difficulty of penetration required that a high dosage of JHAs be used in order to block the distention.

When methoprene was applied to the pupae at P1, successful distention appeared in approximately half of the treated pupae (Fig. 1). However, methoprene did not suppress rectal sac distention when applied to the pupae at P2, indicating that rectal sac responsiveness to JHA is lost between stages P1 and P2. The hemolymph ecdysteroid level increases from P1, reaches a higher level at P2, and keeps a constant level until P4 [2]. When the pupae were applied methoprene at P0, the hemolymph ecdysteroid titer did not reach a level high enough for distention to occur at P2 (Fig. 2). Methoprene application at P0 thus suppressed ecdysteroid elevation at P2. However, rectal sac distention appeared normally when the pupae were applied methoprene at P2 (Fig. 1). At this stage, the hemolymph ecdysteroid titer may have already

reached a level high enough to induce rectal sac distention.

In pupae applied with methoprene, rectal sac distention occurred at P7 (Fig. 3). Around stage P7, more than 10 μM of ecdysteroid was detected in the hemolymph (Fig. 2), suggesting that the delayed distention was due to a delayed increase in ecdysteroid titer. However, 20E administration did not restore completely the failure of rectal sac distention in pupae applied with methoprene (Fig. 4). These conflicting results indicate that methoprene alters changes in the ecdysteroid titer and also inhibits 20E action. In *M. sexta*, 20E and JH synergistically encourage larval prothoracic glands to increase steroidogenic activity [10]. In the bamboo borer, *Omphisa fuscidentalis*, JH activates ecdysteroid synthesis in the prothoracic glands [11]. However, in the present study, JHA treatment delayed pupal-adult development, altered changes in the ecdysteroid titer, and interrupted 20E action.

In pupae applied with methoprene, the hemolymph ecdysteroid titer continued to increase until P6 (Fig. 2). This continued increase in ecdysteroid may be caused by continuous ecdysteroid synthesis in the prothoracic glands and inhibition of ecdysteroid degradation by JHA. In *M. sexta*, JH-II inhibits programmed cell death in the prothoracic glands and maintains steroidogenic activity in the prothoracic glands [12]. However, in *B. mori*, the prothoracic

glands of pupae treated with fenoxycarb reduced steroidogenic activity throughout adult development [13]. Even when the prothoracic glands synthesized a smaller amount of ecdysteroid, the hemolymph ecdysteroid titer did not decrease (Fig. 2 and [13]). The non-decreasing titer may be caused by defects of ecdysteroid degradation and uptake into cells; consequently, ecdysteroid may accumulate in the hemolymph.

We propose a model for hormonal regulation of rectal sac distention by 20E and JHA in *B. mori* (Fig. 6). Prothoracicotropic hormone is secreted by the brain to activate ecdysteroid synthesis in the prothoracic glands. The hemolymph ecdysteroid titer reaches a level high enough to induce rectal sac distention at P2. Consequently, distention appears at P5 (Fig. 6; [2]). In the presence of JHA, the timing of ecdysteroid elevation is delayed and the action of 20E is blocked to delay the timing of rectal sac distention. Thus, in the case of rectal sac distention, JHA operates as a "status quo" action by delaying the timing of ecdysteroid elevation and reducing the effects of 20E as seen in the molting of *M. sexta* [4]. JHA no longer delays distention at P2 because the hemolymph ecdysteroid titer reaches a level high enough to induce distention (Fig. 6). JHA application at P0 inhibited adult emergence completely but that at P2 did not, indicating that JHA caused the inhibition in a stage-specific manner. When JHA is used

as an insect growth regulator, it should be applied to early pupal stage because the response to JHA varies during the stages P0 and P2.

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Table 1. Suppression of rectal sac distention by application of methoprene or fenoxycarb.

Chemicals	Amount	Total number	Distention	No distention	Dead
Methoprene	0 μg	22	20	2	0
	1 μg	18	18	0	0
	10 μg	19	19	0	0
	100 μg	18	15	3	0
	1 mg	16	15	1	0
	5 mg	18	1	16	1
Fenoxycarb	1 μg	15	12	3	0
	10 μg	14	10	4	0
	50 μg	19	19	0	0
	100 μg	15	15	0	0
	1 mg	7	3	4	0

All numerals indicate the number of pupae except the amount of applied chemicals. An amount of 0 μg indicates the application of a solvent, acetone.

Table 2. Stage-specific inhibition of adult emergence by methoprene.

Stage	Methoprene	Total number	Eclosion	Dead
P0	0 mg	20	20	0
P0	5 mg	23	0	23
P2	5 mg	48	29	19

All numerals indicate the number of pupae except the amount of applied methoprene. An amount of 0 mg indicates the application of a solvent, acetone. Stages are the day of application. When the pupae did not move after being touched with forceps, they were judged to be dead.

Figure Legends

Fig. 1. Rectal sac distention was suppressed by JHA in a stage-specific manner. The pupae were applied 5 mg/g of methoprene (closed bar) or acetone (open bar) at one stage between P0 – P2, as indicated, and dissected at P6. Successful distention is expressed as a percent ratio of the number of pupae that show distended sacs to the number of total pupae. Each datum is a mean of three independent experiments ± standard deviation (n = 20 – 31).

Fig. 2. Elevation of ecdysteroid titer delayed in JHA-applied pupae. Ecdysteroid was extracted from the hemolymph of the pupae applied with 5 mg/g methoprene (closed circle) or acetone (open circle) at P0. The ordinate and horizontal axes indicate the ecdysteroid level in the hemolymph and the stage, respectively. The ecdysteroid titer was quantified by radioimmunoassay. The concentration of ecdysteroid is presented as the α-ecdysone equivalent. Each datum is a mean of 5 - 6 different quantifications ± standard deviation.

Fig. 3. JHA delayed timing of successful distention. The pupae were applied with 5 mg/g of methoprene at P0 and dissected at one stage from P6 – P9, as indicated. Successful distention is

expressed as a percent ratio of the number of pupae that show distended sacs to the number of total pupae. Each datum is a mean of 3 – 4 independent experiments ± standard deviation (n = 27 – 53). Asterisks indicate significant differences as compared with the ratio in P6 by a Student's t-test (*$P < 0.05$; **$P < 0.01$).

Fig. 4. JHA interrupted 20E-induced rectal sac distention. The pupae were applied with 5 mg/g of methoprene (closed bars) or acetone (open bars) at P0, injected with 20E at P1, and dissected at a stage from P4 – P6, as indicated. 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 = 24 – 35).

Fig. 5. JHA interrupted 20E action in rectal sac distention. The pupae were brain-removed just after pupal ecdysis, applied with 5 mg/g methoprene (closed bars) or acetone (open bars) at P1, and injected with 1.0 or 3.0 μg/g 20E at P2. The pupae were then dissected at P8. 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

= 21 – 34). Asterisks indicate significant differences between the ratio in acetone and methoprene applied pupae by a Student's t-test (*$P < 0.05$; **$P < 0.01$).

Fig. 6. A model of hormonal regulation of rectal sac distention. In normal development, prothoracicotropic hormone (PTTH) is secreted from the brain just after larval-pupal ecdysis. PTTH stimulates the prothoracic glands to produce ecdysone; consequently, ecdysteroid titer increases to a level high enough to induce rectal sac distention [2]. When P0 or P1 pupae are applied with JHA, the timings of ecdysteroid elevation and distention are delayed as a consequence of the "status quo" action of JHA. In the P2 pupae, JHA no longer blocks rectal sac distention and adult emergence. White, hatched, and gray squares indicate that the responsive, transitional, and unresponsive phases of JHA, respectively.

Figure 1

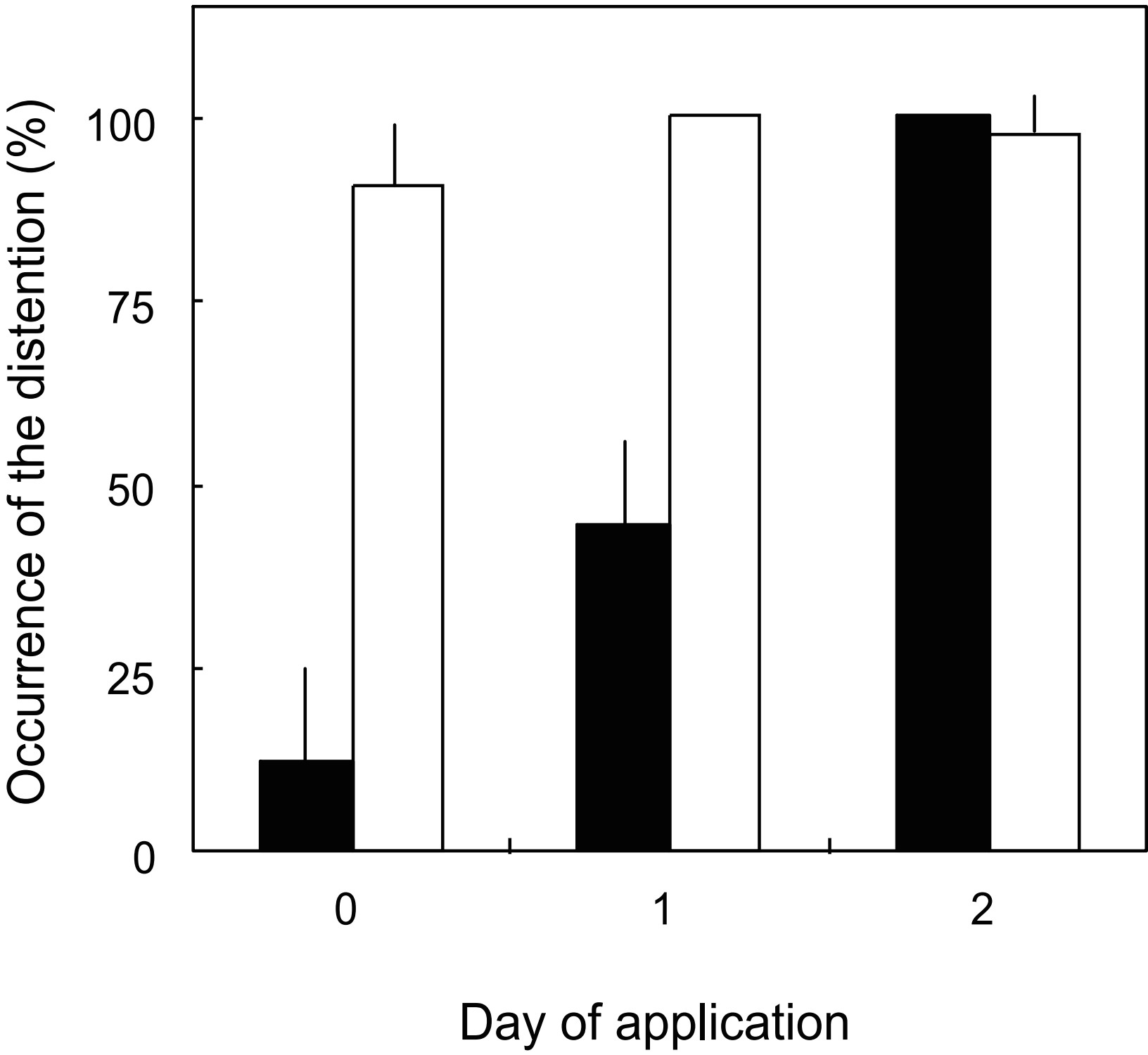

Occurrence of the distention (%)
100
75
50
25
0
0
1
2
Day of application

Figure 2

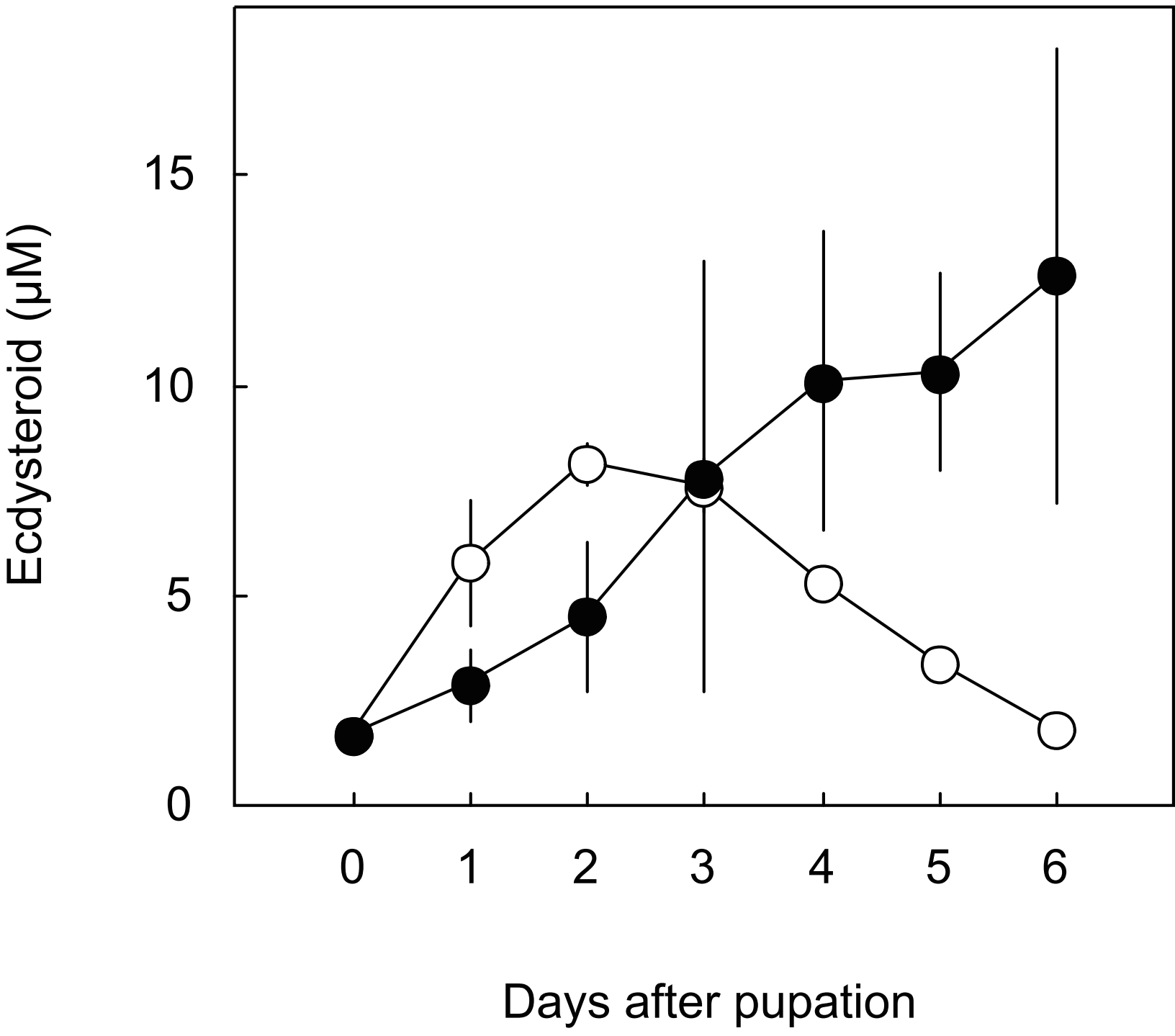


Figure 3

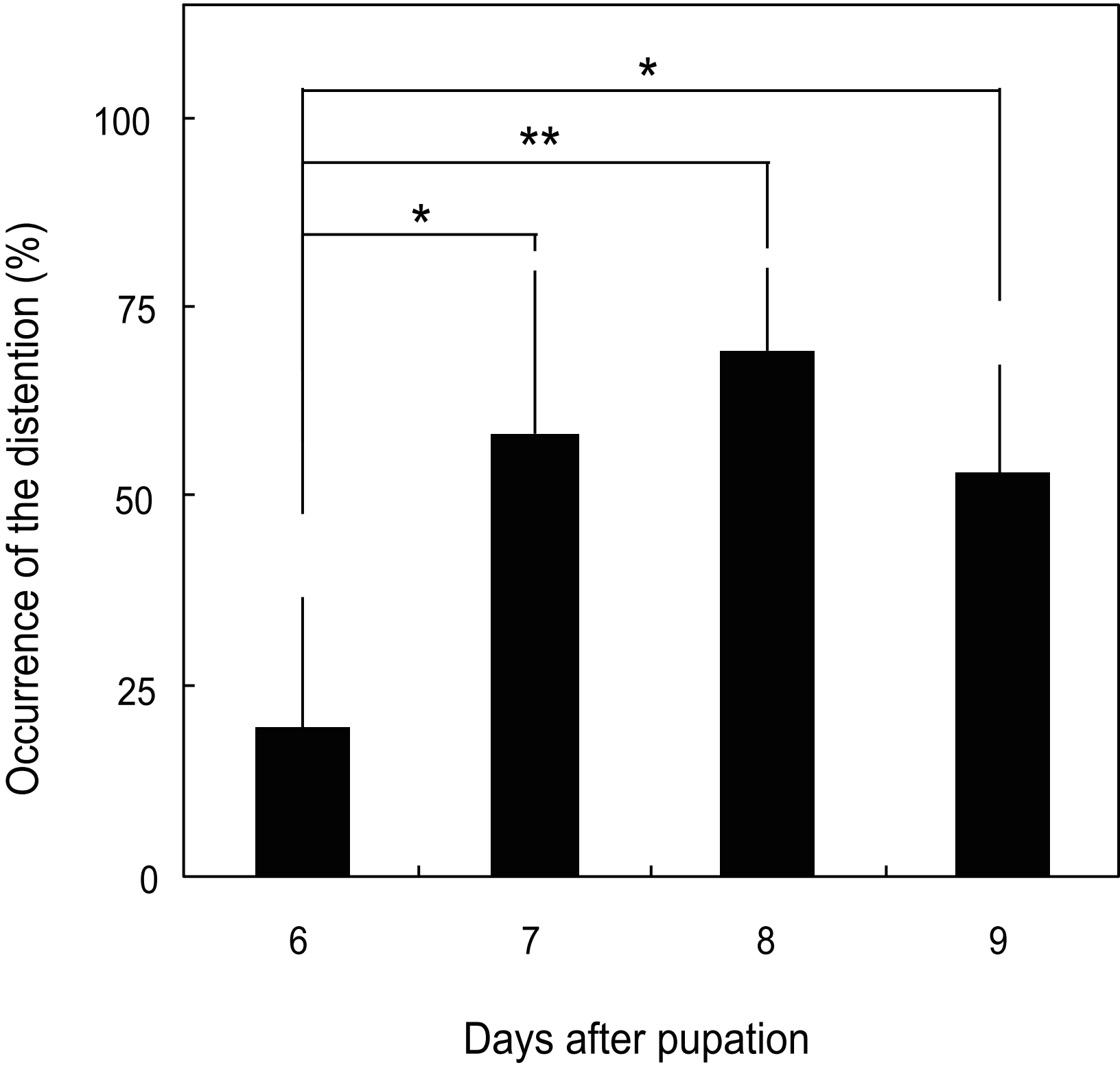

*
**
*
100
75
50
25
0
Occurrence of the distention (%)
6
7
8
9
Days after pupation

Figure 4

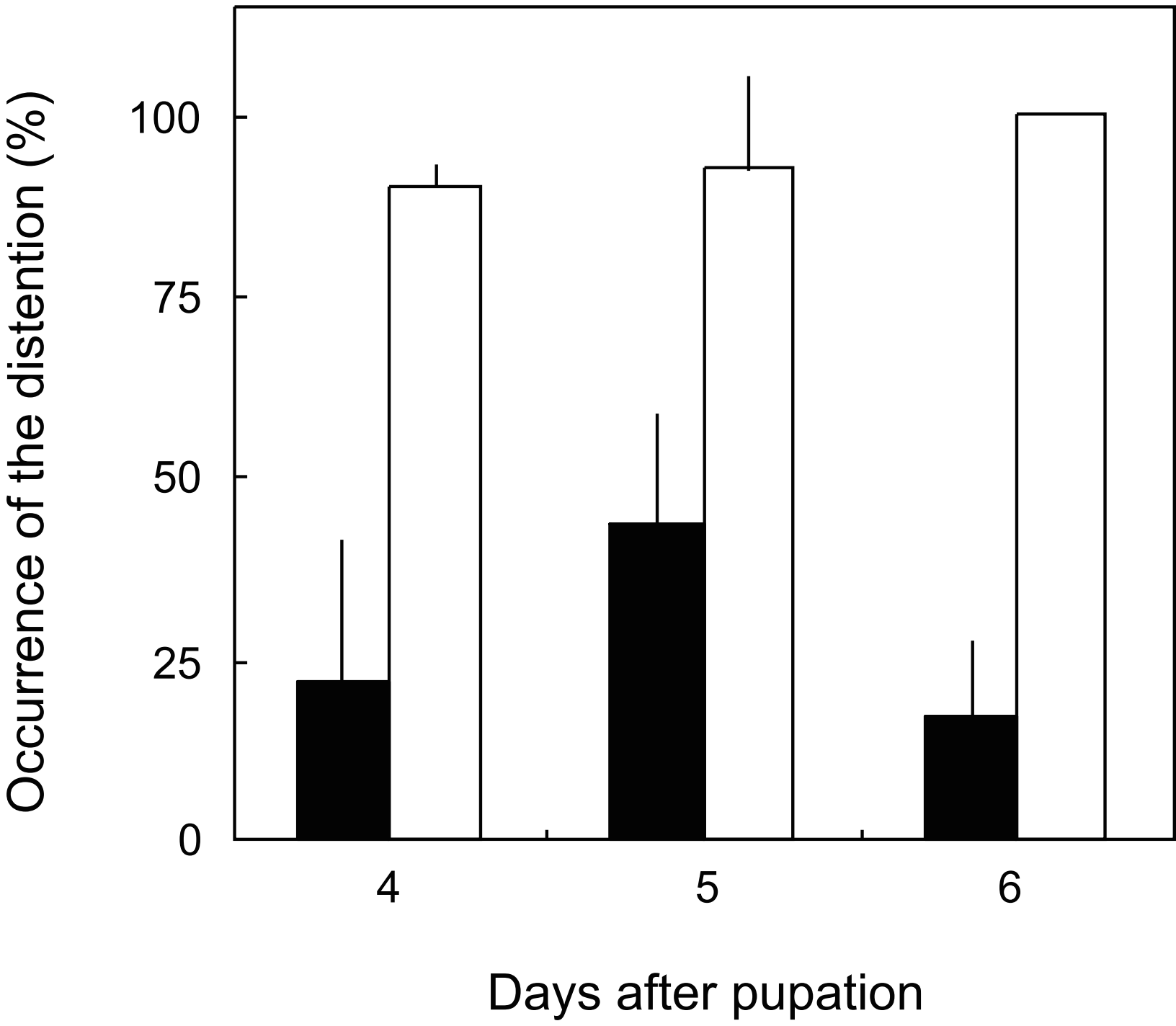

Occurrence of the distention (%)
100
75
50
25
0
4
5
6
Days after pupation

Figure 5

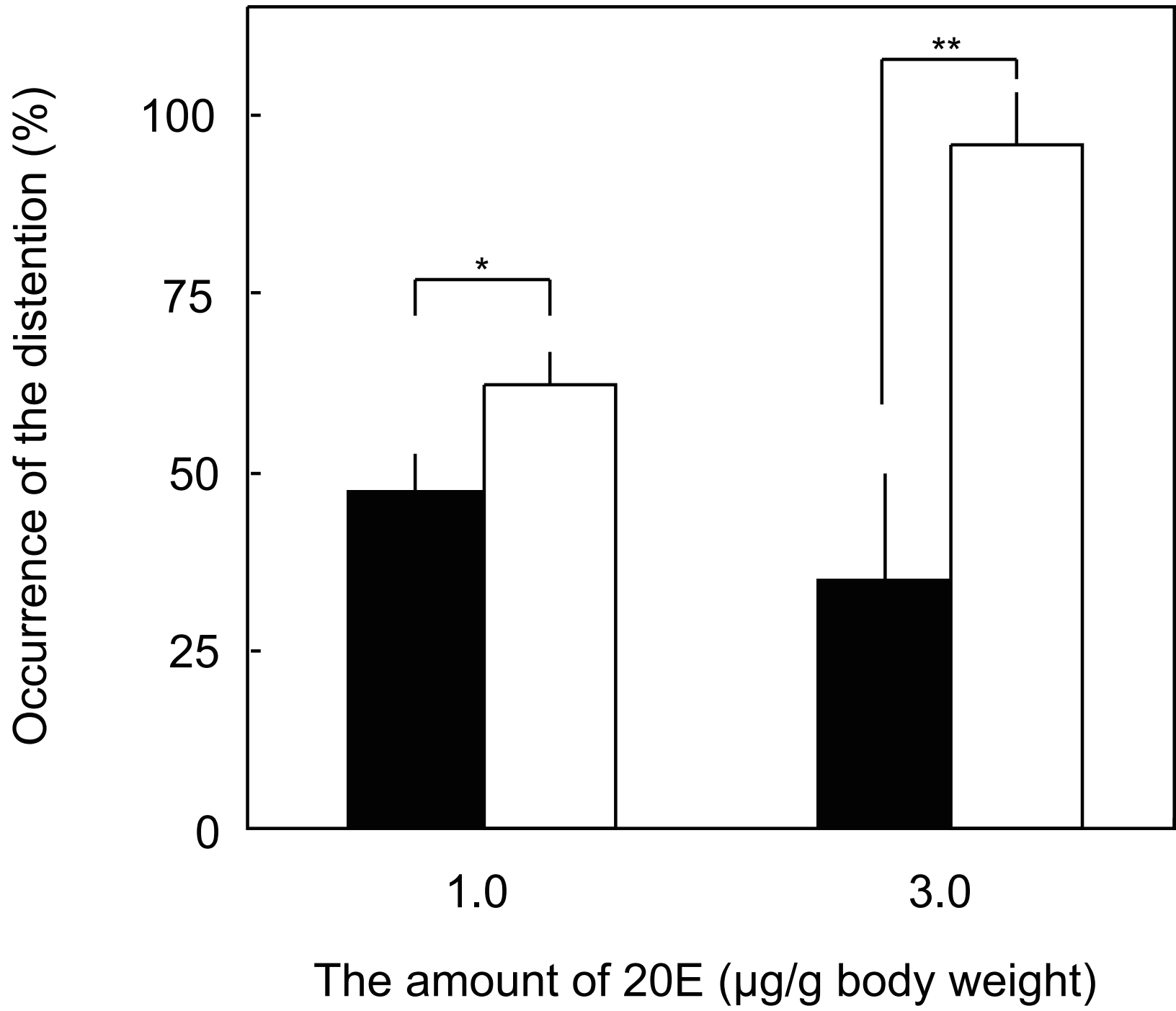
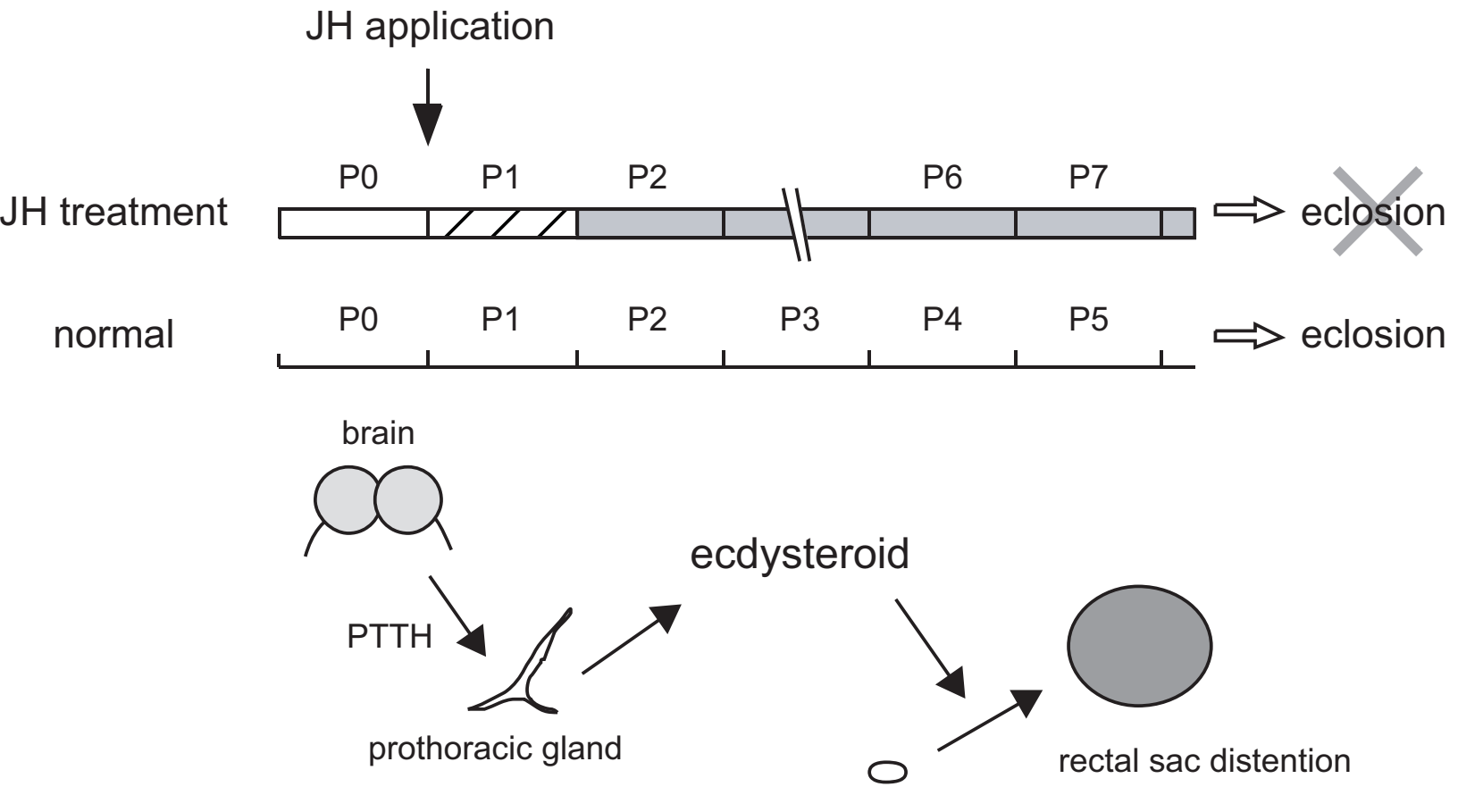

Occurrence of the distention (%)
100
75
50
25
0
*
**
1.0
3.0
The amount of 20E (μg/g body weight)

Figure 6


JH application
JH treatment
P0
P1
P2
P6
P7
eclosion
normal
P0
P1
P2
P3
P4
P5
eclosion
brain
PTTH
prothoracic gland
ecdysteroid
rectal sac distention