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Physiological requirements for 20-hydroxyecdysone-induced rectal sac distention

in the pupa of the silkworm, *Bombyx mori*

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17

1 **Abstract**

2   Successful insect development is achieved via appropriate fluctuation of ecdysteroid

3 levels. When an insect's ecdysteroid level is disrupted, physiological and developmental defects

4 occur. In the pupa of the silkworm, *Bombyx mori*, the rectal sac is an essential organ that

5 operates as a repository for degraded ecdysteroids, and it can be distended by administration of

6 20-hydroxyecdysone (20E). Our previous study showed that rectal sac distention appears 4 days

7 after 20E administration. Hemolymph ecdysteroid levels, however, decrease to lower level

8 during this period. Thus, the timing of the rectal sac distention does not match with that of

9 ecdysteroid elevation. Here, we examine how 20E induces rectal sac distention. A ligature

10 experiment and ecdysteroid quantification showed that continuous 20E stimulation induces

11 rectal sac distention. Thorax tissue contributed to the continuous 20E stimulation needed to

12 induce distention. Ecdysteroid released from the thorax tissue may be converted to 20E by

13 ecdysone 20-hydroxylase to produce continuous 20E stimulation. Thus, the ecdysone metabolic

14 pathway plays a critical role in rectal sac distention.

15

16 **Keywords**: excretory system, ecdysteroid, pupa, metamorphosis, rectal sac

17

1. Introduction

Ecdysteroids play critical roles in the regulation of insect development, and their titers in hemolymph are inseparably connected to developmental events. The hemolymph ecdysteroid titer is controlled by four steps: ecdysteroidogenesis in and release from the prothoracic glands, ecdysteroid uptake into target cells, ecdysteroid degradation at midgut, and excretion of the degraded ecdysteroid through the alimentary canal. Ecdysteroidogenesis in the prothoracic glands is activated by the prothoracicotropic hormone produced in the brain (Gilbert, 2004). When the brain is removed from the silkworm, *Bombyx mori*, the developmental sequence is arrested (Ichikawa and Ishizaki, 1961). In brain-removed pupae, the hemolymph ecdysteroid titer does not elevate and remains constant at a low level (Suzuki *et al*., 2009).

Insects show developmental defects when the ecdysteroid titer is disrupted. The larvae of *B. mori* fail to pupate when the ecdysteroid titer is decreased by ligature between the thorax and abdomen (Fukuda, 1944). By contrast, α-ecdysone, 20-hydroxyecdysone (20E), and inokosterone, a phytoecdysteroid, promote larval-pupal intermediates at high concentrations (Williams, 1968). A non-decreasing 20E titer inhibits both larval-pupal ecdysis and adult eclosion in the mealworm, *Tenebrio molitor* (Sláma, 1980) and the tobacco hornworm,

1   *Manduca sexta* (Truman *et al.*, 1983). The inhibition may be due in part to the suppression of

2   eclosion hormone release (Schwartz and Truman, 1983) and the consequent ecdysis triggering

3   hormone release from Inka cells (Kingan and Adams, 2000). A decrease of the ecdysteroid titer

4   is therefore necessary for successful ecdysis, adult eclosion, and further adult development

5   (Schwartz and Truman, 1982); this is achieved by the degradation and excretion of ecdysteroid

6   at each specific developmental stage.

7   Degraded ecdysteroid is transiently deposited in the rectal sac as meconium

8   (Thompson *et al.*, 1974). In *B. mori*, distention of the rectal sac occurs 4–5 days after pupation

9   (Suzuki *et al.*, 2009). Although distention can be induced by an injection of 20E, the ecdysteroid

10   titer gradually decreases to a basal level in brain-removed pupae. Distention appears when the

11   ecdysteroid titer decreases. Thus, the timing of rectal sac distention does not match that of

12   ecdysteroid elevation. In the present study, we examined the physiological requirements for

13   20E-induced rectal sac distention. Our results indicate that tissue in the thorax is essential for

14   20E-induced rectal sac distention because it causes continuous 20E stimulation. In addition,

15   they suggest that 20-hydroxylation of α-ecdysone also contributes to continuous 20E

16   stimulation.

1

2 **2. Materials and methods**

3 *2.1 Animals*

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

5 Nihon Nosan Kougyo, Yokohama, Japan) at 25 ± 1°C under a 12 h light:12 h dark photoperiod.

6 For our purposes, larvae a day before pupation are called prepupae. The day of pupation is

7 designated day 0 (P0). One day after pupation and 2–8 days after pupation are designated stages

8 P1 and P2–P8, respectively. Also, pupae just after larval-pupal ecdysis are called white pupae

9 (WP).

10

11 *2.2 Hormones and chemicals*

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

13 ethanol and water, respectively. [^{3}H]-Ecdysone (Perkin Elmer, Boston, MA) was dissolved in

14 borate buffer (100 mM boric acid, 50 mM borax, 60 mM NaCl). RH-5992, a non-steroidal

15 ecdysone agonist (the kind gift of Y. Nakagawa of Kyoto University) was dissolved in dimethyl

16 sulfoxide (DMSO). 20E, RH-5992, and DMSO were diluted with insect Ringer's solution (128

1   mM NaCl, 4.7 mM KCl, 1.9 mM CaCl$_2$) for injections. Each chemical, except for α-ecdysone

2   and [^{3}H]-ecdysone, was injected with various dosages per gram body weight.

3

4   *2.3 Operation, hormonal treatment, and observations*

5   Prepupae and WP pupae were ligated between head and thorax or thorax and

6   abdomen by cotton thread followed by excision of the anterior remainder (referred to as isolated

7   abdomens in the text). Two days after pupation, the isolated abdomens were injected with 3.0

8   μg/g 20E solution, 3.0 μg/g RH-5992 solution, or DMSO. Treated pupae were dissected at P8.

9   Brain removal from WP pupa was performed as previously described (Suzuki *et al.*, 2009). The

10   pupae with brain-removed at WP were injected with 3.0 μg/g RH-5992 solution, DMSO, or the

11   same volume of insect Ringer's solution. The wound made by the operation or injection was

12   sealed with melted paraffin wax. Rectal sac distention was observed as previously described

13   (Suzuki *et al.*, 2009).

14

15   *2.4. Quantification of ecdysteroid titer*

16   Hemolymph was collected from the pupae through an incision on the dorsal side.

1   Ecdysteroids were extracted from the hemolymph and quantified by radioimmunoassay as

2   previously described (Sakurai *et al*., 1998). Anti-ecdysone antiserum H-22 was obtained from L.

3   I. Gilbert and D. H. S. Horn and used as a capture antibody in the radioimmunoassay (Warren

4   and Gilbert, 1986). We examined the cross-reactivity of anti-ecdysone antiserum H-22 against

5   RH-5992; no cross-reactivity was found (data not shown).

6

7   *2.5. Reverse transcription (RT)-PCR*

8   Total RNA was extracted from pupal midgut by Trizol reagent (Invitrogen) and then

9   treated with DNase I (Promega, Madison, WI). Complementary DNA was prepared from 0.6 μg of

10   total RNA with reverse transcriptase ReverTra Ace (Toyobo, Osaka, Japan) by an anchored oligo-dT

11   according to the manufacture's protocol. The PCR primer were designed according to the nucleotide

12   sequences: *ribosomal   protein   L3   (RpL3)*,   5'-AGCACCCCGTCATGGGTCTA-3'   and

13   5'-TGCGTCCAAGCTCATCCTGC-3';   *ecdysone   receptor   isoform   A   (EcR-A)*,

14   5'-TGGAGCTGAAACACGAGGTGGC-3'   and   5'-TCCCATTAGGGCTGTACGGACC-3';

15   *ecdysone   Receptor   isoform   B1   (EcR-B1)*,   5'-ATAACGGTGGCTTCCCGCTGCG-3'   and

16   5'-CGGTGTTGTGGGAGGCATTGGTA-3';   *ultraspiracle   1   (Usp-1)*,

1   5'-GTCGAGCGTGGCGAAGAAA-3'   and   5'-CAGCCATTGTATATCGAGTTCAA-3';

2   *Ultraspiracle 2 (Usp-2)*,   5'-GATATCGTGATAATAAACCTAAGTA-3'   and

3   5'-GCAACAAGGTCGTTGAACTAA-3';   *shade*,   5'-TTGTAGCTGTAACCGCGTTG-3'   and

4   5'-GTACGGCCTCAAGAGCAGTC-3'. These primers were designed according to previous report

5   (Kaneko *et al*., 2006) except primers for *shade* (Maeda *et al*., 2008). PCR was carried out with Go

6   Taq (Promega) under following conditions: denature at 94 ˚C for 30 seconds, annealing at different

7   temperatures for each gene (*RpL3*, *EcR-A*, and *EcR-B1*: 60˚C; *Usp-1*: 62˚C; *Usp-2*: 56˚C; *shade*:

8   57˚C) for 30 seconds, and extension at 72 ˚C for 30 seconds, followed by 1 minute as a final

9   extension. *RpL3* was used as an internal standard and amplified for 30 cycles. *EcR-A*, *EcR-B1*,

10   *Usp-1*, *Usp-2*, and *shade* were amplified for 35, 35, 37, 37, and 33 cycles, respectively. The products

11   were cloned into pGEM-T vector (Promega) and subjected to sequence analysis. The sequences of

12   these plasmids were expected ones, indicating that each sequence was correctly amplified under the

13   present experimental condition.

14

15   **3. Results**

16   *3.1 The thorax was required for rectal sac distention induced by 20E*

1   Distention appears 4 days after 20E injection in brain-removed pupae (Suzuki *et al.*,

2   2009). When we monitored the ecdysteroid titer, by the fourth day it had fallen below 3.0 μM

3   (Fig. 1). Although rectal sac distention is induced by 20E (Suzuki *et al.*, 2009), the timing of

4   successful distention does not coincide with that of ecdysteroid elevation. There are two

5   possible explanations for this time lag: either 20E elevation triggers the initiation of distention,

6   but distention requires 4 days to take place, or the ecdysteroid decrease that follows the

7   elevation induces distention. To distinguish between these hypotheses, we prepared two

8   different pupae: brain-removed and ligated pupae. WP pupae were either brain-removed or

9   ligated between thorax and abdomen (isolated abdomen), injected with 3.0 μg/g 20E or insect

10   Ringer's solution, and dissected at P6. An injection of 20E successfully induced distention in

11   87% of the brain-removed pupae and in 20% of the isolated abdomens (Table 1). In the control

12   experiment, insect Ringer's solution did not induce distention.

13   We then used pupae ligated a day before pupation. Prepupae were ligated between

14   head and thorax (decapitated) or between thorax and abdomen, then injected with 3.0 μg/g 20E

15   or insect Ringer's solution and dissected at P6 and P8. At P6 and P8, 24% and 53%, respectively,

16   of the decapitated pupae injected with 20E exhibited distention (Table 2). Distention did not

1   appear in the decapitated pupae injected with insect Ringer's solution. The isolated abdomens

2   injected with 20E did not show distention at either P6 or P8. Thus, the decapitated pupae

3   exhibited distention as the result of an injection of 20E, but the isolated abdomens did not

4   exhibit any, indicating that the thorax was necessary to successfully achieve distention.

5

6   *3.2 Continuous stimulation of 20E was required for successful distention*

7   Injection of 20E restored rectal sac distention in the decapitated pupae but not in the

8   isolated abdomens (Table 2). Decapitated pupae contain a larger amount of ecdysteroid than

9   isolated abdomens because the prothoracic glands produce ecdysteroid in the pupae, indicating

10   that successful distention requires continuous exposure to ecdysteroid. Isolated abdomens do

11   not contain sufficient endogenous ecdysteroid probably because they lack prothoracic glands

12   and cannot achieve successful development without exogenous ecdysteroid. 20E may expire

13   easily, since injected 20E has a half-life as short as 4 hours (Nijhout, 1976). We therefore

14   attempted to give isolated abdomens multiple injections of 20E. Abdomens isolated at the

15   prepupal stage were injected with 0.25–0.5 μg/g 20E during P2–P4 and dissected at P8. Double

16   injections of 0.5 μg/g 20E at P2 and P3 and triple injections of 0.25 μg/g 20E every P2–P4 did

1   not restore distention (Table 3). By contrast, triple injections of 0.5 μg/g 20E significantly

2   restored distention (97%), indicating that induction of distention in isolated abdomens requires

3   continuous stimulation by 20E.

4

5   *3.3 The thorax was necessary to keep the ecdysteroid level high enough to induce distention*

6   Table 3 indicates that decapitated pupae required a 20E injection, but isolated

7   abdomens required triple injections for successful rectal sac distention. These results imply that

8   injected 20E was degraded in the isolated abdomens. We quantified the hemolymph ecdysteroid

9   titer in both decapitated pupae and isolated abdomens injected with 20E. The decapitated pupae

10   and isolated abdomens were injected with 3.0 μg/g of 20E or insect Ringer's solution at P2, and

11   hemolymph was collected during P3–P8. The ecdysteroid titers during P1–P2 were 1.11 ± 0.26

12   $- 1.26 \pm 0.20$ μM and $0.45 \pm 0.08 - 0.92 \pm 0.20$ μM in the decapitated pupae and isolated

13   abdomens, respectively (Fig. 2). After an injection with 20E, ecdysteroid titers were

14   significantly elevated in both the decapitated pupae and isolated abdomens, and they remained

15   at 4.4–5.2 μM and 3.2–3.7 μM, respectively. Insect Ringer's solution did not cause any change

16   in the ecdysteroid titer. Thus, the hemolymph ecdysteroid titer in the decapitated pupae

1   remained at a higher level than that in the isolated abdomens. This higher ecdysteroid level may

2   be sufficient for achievement of continuous stimulation of 20E in the decapitated pupae.

3

4   *3.4 Rectal sac distention was induced by non-degradative ecdysone*

5   We confirmed that continuous stimulation by 20E induces rectal sac distention in the

6   decapitated pupae by using a non-degradative ecdysone agonist, RH-5992. RH-5992 has a

7   longer half-life than 20E (Retnakaran *et al*., 1995) and did not affect *de novo* ecdysteroid

8   synthesis (Fig. 3). The effects of RH-5992 on rectal sac distention can be examined without

9   interference from endogenous ecdysteroid. Hemolymph was collected from pupae injected with

10   RH-5992 or DMSO at P4 and P5. There was no statistically significant difference between the

11   ecdysteroid titer of pupae injected with RH-5992 and that of the pupae injected with DMSO at

12   either stage. Pupae were brain-removed at the WP stage, injected with 3.0 μg/g RH-5992 at P2,

13   and dissected at P8. As a control, insect Ringer's solution and DMSO were also injected. After

14   an injection of RH-5992, 75% of the pupae showed successful distention (Table 4). No pupae

15   exhibited distention as a result of injection with insect Ringer's solution or DMSO.

16   In isolated abdomens, successful distention was only achieved by triple injections of

1   20E (Table 3). A single injection of RH-5992 induced distention in isolated abdomens. RH-5992

2   injected at P2 induced rectal sac distention in 58% of the isolated abdomens by P8 (Table 5).

3   DMSO did not induce distention in the isolated abdomens. Thus, a single injection of

4   non-degradative ecdysone agonist induced rectal sac distention in isolated abdomens, indicating

5   that successful distention requires continuous ecdysteroid stimulation and does not require a

6   decrease in ecdysteroid titer.

7

8   *3.5 Ecdysone receptor genes and ecdysone 20-hydroxylase gene were expressed in the midgut*

9   We considered the reason why single injection of 20E was able to induce rectal sac

10   distention in the decapitated pupae. An injection of 20E stimulates ecdysone 20-hydroxylase

11   activity in the midgut of *M. sexta* (Keogh *et al*., 1989) and *Spodoptera littoralis* (Williams *et al*.,

12   1997). We therefore examined 20E effects on expression of ecdysone 20-hydroxylase gene in

13   the pupal midgut. First, expression levels of ecdysone receptors were examined by

14   semi-quantitative RT-PCR to confirm that 20E acted in the midgut. The expressions of *EcR-A*

15   and *EcR-B1* were kept at constant levels throughout P0 – P6 (Fig. 4). Similarly, *usp-1* and *usp-2*

16   were also expressed at constant levels during P0 – P6. Throughout the periods, 20E was able to

1   act in the midgut since ecdysone receptor genes were expressed in the tissue constantly. Second,

2   we examined the expression profile of ecdysone 20-hydroxylase gene, *shade* (Petryk *et al.*,

3   2003; Maeda *et al.*, 2008) and found that the level of *shade* expression in the midgut during P0

4   – P6. The gene *shade* was thus expressed at constant level during examined periods (Fig.4).

5

6   **4. Discussion**

7   In this study, we explored the physiological requirements for 20E-induced rectal sac

8   distention, and we found that thorax tissue is required. An injection of 3.0 μg/g 20E induced

9   rectal sac distention in over 50% of decapitated pupae, but it did not induce distention in

10   isolated abdomens (Table 3), indicating that the thorax is necessary for rectal sac distention.

11   Triple injections of over 0.5 μg/g 20E or a single injection of RH-5992 successfully induced

12   distention in isolated abdomens (Tables 3, 5). This result indicates that continuous ecdysteroid

13   stimulation is necessary for distention to occur. In decapitated pupae, continuous 20E

14   stimulation can be achieved by a single injection, while a triple injection of 20E or an injection

15   of RH-5992 can induce distention in isolated abdomens. For continuous ecdysteroid stimulation,

16   injected 20E could stimulate ecdysteroid synthesis in thorax tissue. Exogenous 20E stimulates

14

1   ecdysteroid synthesis in the prothoracic glands in the pupae of *M. sexta* (Sakurai and Williams,

2   1989). Regardless of the presence of prothoracic glands, an injection of 20E induced rectal sac

3   distention in 87% of brain-removed pupae (Table 1) and in only 24% of decapitated pupae at P6

4   (Table 3). The different responsiveness may be due to a change of 20E action to prothoracic

5   glands during metamorphosis (Sakurai and Williems, 1989).

6   The gene encoding ecdysone 20-hydroxylase, enzyme that promotes conversion of

7   α-ecdysone to 20E (Gilbert, 2004), was expressed constantly in the midgut during P0 – P6 (Fig.

8   4). Ecdysteroid from the prothoracic glands may contribute to inducing rectal sac distention by

9   acting as a source of 20E, since an injection of 20E induced rectal sac distention in the

10   decapitated pupae but did not in the isolated abdomens. In fact, the ecdysteroid titer in

11   decapitated pupae was almost 2-fold higher than that in isolated abdomens (Fig. 2). Thus,

12   endogenous α-ecdysone may contribute to continuous 20E stimulation to successfully induce

13   distention.

14   Injected 20E degenerates rapidly in the larvae of *M. sexta*, and the half-life of 20E is

15   approximately 4 hours in isolated abdomens (Nijhout, 1976). We previously reported that the

16   ecdysteroid titer decreased to a basal level 4 days after injection of brain-removed pupae with

1 20E (Suzuki *et al*., 2009). However, in decapitated pupae and in isolated abdomens, hemolymph

2 ecdysteroid titers remained at constant levels after 20E injection (Fig. 2). The ecdysteroid titers

3 were quantified by radioimmunoassay using anti-ecdysone antiserum as a capture antibody. The

4 antiserum recognizes both active and inactive ecdysteroid (Warren and Gilbert, 1986). The

5 detected ecdysteroids could be a mixture of active and inactive ecdysteroids because rectal sac

6 distention required continuous 20E stimulation and distention was not induced in isolated

7 abdomens (Table 2, 3). Thus, 20-hydroxylation may be required for continuous stimulation to

8 successfully induce distention. Although it is not clear what causes the different ecdysteroid

9 titers between brain-removed pupae and ligated pupae after 20E injection, our results suggest

10 that the ecdysteroid metabolic pathway is involved in the sequence of 20E-induced rectal sac

11 distention.

12

5

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3

1   **Figure legends**

2

3   Fig.1. Rectal sac distention and hemolymph ecdysteroid titer. (A) Pupae were dissected between

4   P3 and P6. Rectal sacs distended between P5 and P6, and the ecdysteroid titer (open circle)

5   started to decrease at P5. (B) Pupae were brain-removed at WP stage and injected with 3.0 μg/g

6   20E solution 2 days after brain removal. Sacs distended 3–4 days after injection, and the titer

7   (open circle) started to decrease 3 days after injection. Successful distention is expressed as the

8   ratio of the number of pupae that show distended sacs to that of total pupae. The arrow indicates

9   the day of 20E injection. Each datum indicates the mean ± standard deviation. Data are taken

10   and modified from Suzuki *et al*., 2009.

11

12   Fig. 2. Ecdysteroid titers in normal pupae, decapitated pupae, and isolated abdomens. Prepupae

13   were ligated between head and thorax (decapitated pupae, closed circles) or thorax and

14   abdomen (isolated abdomens, triangles) and injected with 3.0 μg/g of 20E solution 2 days after

15   pupation. As a control (open circles), decapitated pupae were injected with insect Ringer's

16   solution at P2. The titers in normal pupae are indicated as open squares. Each datum indicates

17   the mean ± standard deviation ($n = 6 – 10$).

23

1

2 Fig. 3. Ecdysteroid titers in brain-removed pupae injected with RH-5992. Pupae were brain-

3 removed at WP stage and injected with 3.0 μg of RH-5992 (closed bars) or DMSO (open bars) 2

4 days after brain removal. Hemolymph was collected from the pupae 4 and 5 days after pupation.

5 The ecdysteroid titer was quantified by radioimmunoassay. The concentration of ecdysteroid is

6 presented as α-ecdysone equivalent. Each datum indicates the mean ± standard deviation (n =

7 8).

8

9 Fig. 4. Temporal expression profiles of *EcR-A*, *EcR-B1*, *Usp-1*, *Usp-2*, *shade*, and *RpL3* in the

10 midgut during P0 – P6. The gene *RpL3* was used as internal standard. The term –RT indicates

11 that the RNA samples were subjected to PCR without reverse transcriptional reaction. The

12 expression profiles were analyzed 3 times by independent experiments and show no difference

13 in the experiments.

1 Table 1 20E did not induce rectal sac distention in isolated abdomens of white pupae

Treatment	Chemical	Total	Distention	No distention	Dead	Ratio (%)
Brain removal	Ringer's	15	0	15	0	0
	20E	15	13	2	0	87
Abdominal	Ringer's	13	0	13	0	0
isolation	20E	15	3	12	0	20

2 All numerals indicate the number of pupae except ratios, which are expressed as percentages.

3

1   Table 2 20E induced rectal sac distention in decapitated pupae but not in isolated abdomens

2   treated 1 day before pupation

Stage	Treatment	Total	Distention	No distention	Dead	Ratio (%)
P6	Intact	21	21	0	0	100
	Decapitated + Ringer's	16	0	16	0	0
	Decapitated + 20E	29	7	20	2	24
	Isolated abdomen + 20E	32	0	29	3	0
P8	Decapitated + Ringer's	16	1	13	2	0
	Decapitated + 20E	30	16	14	0	53
	Isolated abdomen + 20E	31	3	25	3	10

3   All numerals indicate the number of pupae except ratios, which are expressed as percentages.

4

1   Table 3 Multiple injections of 20E restored rectal sac distention in isolated abdomens

Dose	Total	Injections	Distention	No distention	Dead
0.25 μg	30	2	4	25	1
0.5 μg	38	2	4	33	1
0.5 μg	35	3	34	0	1

2   All numerals indicate the number of pupae except the 20E dosage and the number of injections.

3

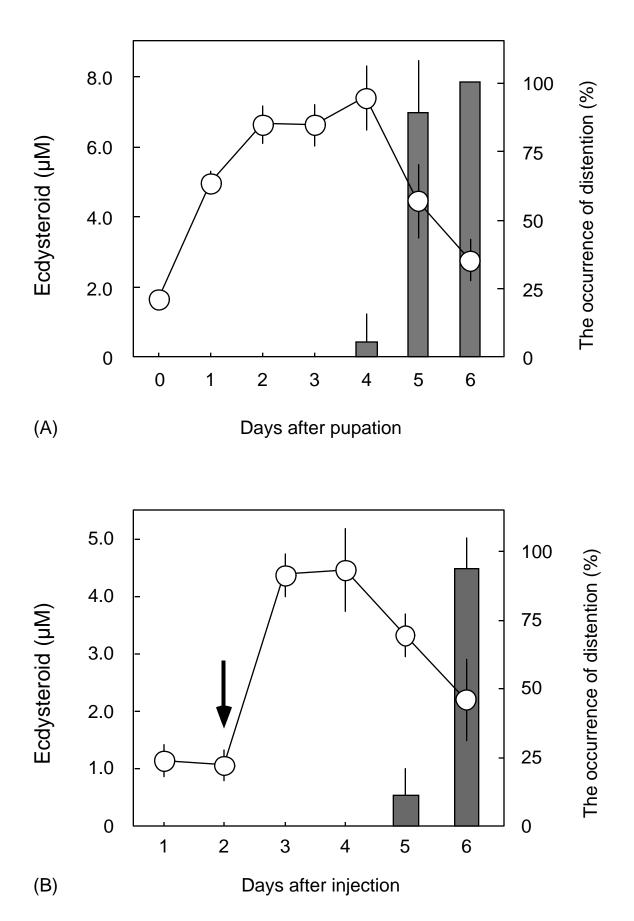1   Table 4 RH-5992 induced rectal sac distention in brain-removed pupae

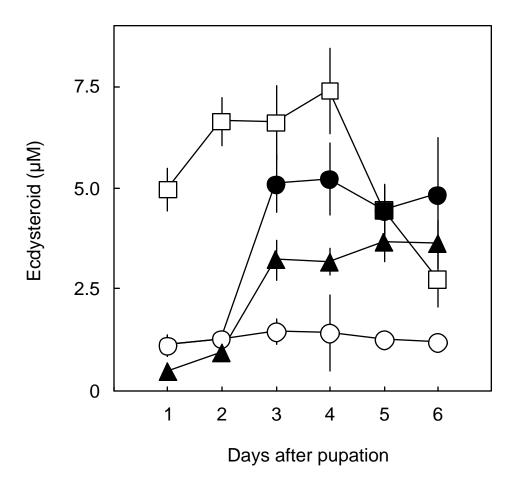| Chemical	Total	Distention	No distention	Ratio (%)
Ringer's	10	0	10	0
DMSO	12	0	12	0
RH-5992	12	9	3	75

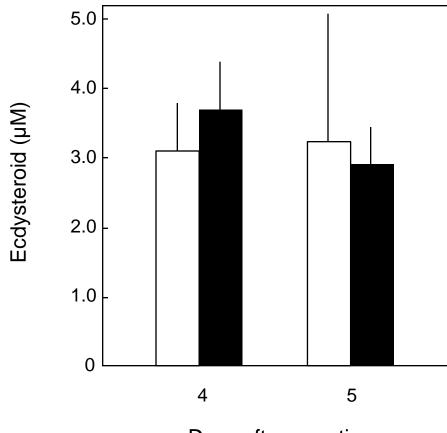2   All numerals indicate the number of pupae except ratios, which are expressed as percentages.

3

1   Table 5 RH-5992 induced rectal sac distention in isolated abdomens

Chemical	Total	Distention	No distention	Dead	Ratio (%)
20E	15	0	15	0	0
DMSO	26	0	26	0	0
RH-5992	31	18	12	1	58

2   All numerals indicate the number of pupae except ratios, which are expressed as percentages.

(A)

Ecdysteroid (µM)

The occurrence of distention (%)

Days after pupation

(B)

Ecdysteroid (µM)

The occurrence of distention (%)

Days after injection