Hormonal regulation of the death commitment in programmed cell death of the silkworm anterior silk glands

Hormonal regulation of the death commitment in programmed cell death of the silkworm anterior silk glands

Hiroto Matsui[1,3], Motonori Kakei[2,4], Masafumi Iwami[1,2], Sho Sakurai[1,2]

[1]Division of Biological Sciences; [2]Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan

[3]Present address: Biological Research Laboratories, Nissan Chemical Industries, Shiraoka, Shiraokamachi, Saitama, 349-0294, Japan

[4]Present address: Translational Medical Center, National Center of Neurology and Psychiatry, Ogawahigashimachi, Kodaira, Tokyo 187-8551, Japan

Corresponding author

Sho Sakurai, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192.

E-mail: ssakurai@staff.kanazawa-u.ac.jp

Phone: +81 76 264 5083

Fax: +81 76 234 4017

Abstract

During larval-pupal transformation, the anterior silk glands (ASGs) of the silkworm *Bombyx mori* undergo programmed cell death (PCD) triggered by 20-hydroxyecdysone (20E). Under standard in vitro culture conditions (0.3 ml of medium with 1 μM 20E), ASGs of the fourth-instar larvae do not undergo PCD in response to 20E. Similarly, larvae of the fifth instar do not respond to 20E through day 5 of the instar (V5). However, ASGs of V6 die when challenged by 20E, indicating that the glands might be destined to die before V6 but that a death commitment is not yet present. When we increased the volume of culture medium for one gland from 0.3 ml to 9 ml, V5 ASGs underwent PCD. We examined the response of ASGs to 20E every day by culturing them in 9 ml of medium and found that ASGs on and after V2 were fully responsive to 20E. Because pupal commitment is associated with juvenile hormone (JH), the corpora allata (a JH secretory organ) were removed on day 3 of the fourth larval instar (IV3), and their ASGs on V0 were cultured with 20E. Removal of the corpora allata allowed the V0 larval ASGs to respond to 20E with PCD. In contrast, topical application of a JH analogue inhibited the response to 20E when applied on or before V5. We conclude that the acquisition of responsiveness to 20E precedes the loss of JH sensitivity, and that the death commitment in ASGs occurs between V5 and 6.

Key words: anterior silk gland; *Bombyx mori*; commitment; ecdysone; juvenile hormone; programmed cell death; glucose oxidase; hydrogen peroxide

Introduction

In insects, the larval-specific tissues are partly or entirely eliminated from the pupal bodies during larval-pupal transformation in response to a metamorphic increase in hemolymph ecdysteroid concentration (von Gaudecker and Schmale, 1974; Chinzei, 1975; Schwartz, 1992; Terashima et al., 2000). In addition, larval tissues respond to 20-hydroxyecdysone (20E), a biologically active type of ecdysteroid, by undergoing various developmental changes. These changes are determined in advance according to the actual responses, and this process is known as pupal commitment (Riddiford, 1985).

Bombyx mori anterior silk gland (ASG) is a larval-specific tissue that is eliminated through programmed cell death (PCD) in response to 20E. This PCD occurs after the onset of cocoon spinning during the latter part of day 5 of the fifth larval instar (V5) (Terashima et al., 2000). Similarly, ASGs after the onset of spinning undergo PCD when treated with 20E in vitro. The ability of ASGs to respond to 20E first appears in some ASGs late V5 and in all on V6, the day of wandering (Kakei et al., 2005).

In pupal commitment, acquisition of responsiveness to 20E is the first step of the change in commitment. JH inhibits this change in commitment, and the commitment is completed by the loss of sensitivity to JH (Riddiford, 1985; Obara et al., 2002; Koyama et al., 2004). In *B. mori* fifth instar ASGs, the loss of sensitivity to JH begins between V4 and V5 of the instar and is completed by V6 (Kakei et al., 2005). However, the ASGs first exhibit responsiveness to 20E late on V5, which is after the beginning of the loss of sensitivity to JH. These observations led us to question whether the commitment to death occurs in ASGs in a manner

similar to that observed in epidermis and imaginal discs.

20E initially binds to ecdysone receptor (EcR) and then sequentially regulates early-response gene expression. This mode of action is referred to as a genomic action of a steroid hormone. In the 20E-induced PCD of the *Drosophila melanogaster* salivary gland, the action of 20E begins with a hierarchical regulation of early-response genes and culminates with the activation of late-response genes. A similar hierarchical expression of early-response genes occurs in the 20E-induced PCD of *B. mori* ASGs (Sekimoto et al., 2006, 2007).

In addition to genomic action, 20E exhibits non-genomic action. Specifically, its downstream effects activate the death effector caspase 3-like protease (Iga et al., 2010). Indirect evidence indicates that the non-genomic action begins with 20E binding to a putative membrane ecdysone receptor, which likely belongs to a family of G-protein-coupled receptor, and activating a signal transduction pathway. This action is followed by the activation of caspase 3-like protease, which completes the PCD via DNA fragmentation (Manaboon et al., 2009).

We recently identified a third factor in the control of 20E-induced PCD; this factor appears in the medium during ASG culture and inhibits the action of 20E (Kakei et al., 2005). This PCD inhibitory factor was identified as glucose oxidase (GOD), which may be produced by ASG cells. The catalytic by-product of GOD, hydrogen peroxide (H_2O_2), is the immediate inhibitory factor (Matsui et al., 2011).

ASGs in vitro do not respond to 20E by undergoing PCD before the onset of spinning. However, this lack of response to 20E is not caused by the glands' own lack of competency to respond to 20E. Rather, the lack of response is a result of the H_2O_2 that is produced by the GOD released into the medium. In addition, the

97 production of H_2O_2 in the medium conceals the involvement of JH in the
98 acquisition of responsiveness to 20E. These multiple factors make it difficult to
99 examine whether ASGs are committed to die (death commitment) before the
100 spinning (Kakei et al., 2005).

101 We therefore developed a simple method to limit the effects of GOD on the
102 ASGs. Specifically, the ASGs were incubated in a large volume of culture medium,
103 which reduced the H_2O_2 concentration to a level at which it had no effect on the
104 ASGs. Using this culture condition, we examined the presence of the death
105 commitment and its timing. At V2 (4 days before the onset of spinning), *B. mori*
106 ASGs respond to 20E by undergoing cell death. At the onset of spinning (late V5 -
107 early V6), however, the glands completely lose their sensitivity to JH. After V2,
108 the JH concentration in the hemolymph is very low, but the glands are regulated
109 not to respond to 20E. These unstable conditions between V2 and V6 are strictly
110 controlled by GOD, which blocks the cell death pathway if ASGs are exposed to
111 high 20E. This study is the first to show the presence of the death commitment in
112 in insect metamorphosis and proposes a unique control mechanism for cell death.
113

114 ## 1. Materials and Methods

115 ### *2.1. Animals*

116 Larvae of the silkworm *B. mori* were reared and staged as previously described
117 (Sakurai et al., 1998). Newly molted fifth instar larvae were fed from the
118 beginning of the photophase following the scotophase during which they molted to
119 fifth instars. The 24 h period of the photophase following the scotophase during
120 which the fourth-instar larva molted was designated day 0 of the fifth instar (V0).

ASGs were dissected during the photophase of each day. Corpora allata (CA) were removed from fourth-instar larvae as described (Sakurai, 1983).

2.2. Hormones and tissue culture

A juvenile hormone analogue (JHA), S-methoprene (95% stereochemically pure; SDS Biotech, Tokyo), was dissolved in acetone, and a 5-μl aliquot was applied to the dorsal surface of individual larvae. 20E (Sigma, St. Louis, MO) was dissolved in distilled water (1 mg/ml) and stored at -20°C. ASGs were rinsed with Grace's insect cell culture medium (Gibco BRL, Rockville, MD) and cultured individually in Grace's medium (pH 6.4, adjusted with NaOH) with or without 1 μM 20E at 25°C. The ASGs were observed every 24 h, and the degree of PCD progression was noted with PCD score according to the changes in their cellular morphology (Terashima et al., 2000; Kakei et al., 2005), where a score of 0 indicates no change, and a score of 6 indicates the completion of PCD.

2.3. Staining

ASGs were fixed with 4% formaldehyde for more than 30 min and washed with phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.47 mM KH_2PO_4, pH 7.4). The samples were then incubated in PBS containing DAPI (0.1 μg/ml) at 25°C in the dark for 15 min. Finally, the samples were washed with PBS and observed under a fluorescence microscope using a UV excitation filter (BX-50, Olympus, Tokyo).

2.4. Reverse transcription (RT)-PCR

145 Total RNA was extracted from ASGs (Chomcyzuski and Sacchi, 1987) and treated
146 with RNase-free DNase (Promega, Madison, WI). Complementary DNA (cDNA)
147 was prepared from 1 μg of total RNA using anchored oligo-dT
148 [5'-(T)12(A/C/G)(A/C/G/T)-3'] and ReverTra Ace reverse transcriptase (Toyobo,
149 Osaka, Japan). For RT-PCR, Krüppel homolog 1 (*Kr-h1*) cDNA was amplified for
150 40 cycles with the following primers: forward, 5'-GCGAGTGTGGTTTGACATTG;
151 reverse, 5'-GATACGGCCTCTCCTTTGTG. RT-PCR targeting 20E-induced genes
152 was performed using the same primer sets as described in Sekimoto et al., 2006.
153 RNA encoding ribosomal protein L3 (*RpL3*) was used as an internal standard and
154 amplified for 25 cycles. PCR products were separated by agarose gel
155 electrophoresis.
156
157 *2.5. DNA isolation and agarose gel electrophoresis*
158 ASGs were homogenized and mixed in DNA extract buffer (10 mM Tris-HCl, 150
159 mM NaCl, 10 mM EDTA-NaOH, 0.1% sodium dodecylsulfate, pH 8.0) on ice. The
160 homogenate was treated with RNase (20 μg/ml, 37°C, 30 min) and proteinase K
161 (100 μg/ml, 50°C, 60 min). DNA was extracted using a standard
162 phenol-chloroform and chloroform extraction method. The DNA was then
163 electrophoresed on a 2% agarose gel in TAE buffer and stained with ethidium
164 bromide.
165
166 *2.6. Hydrogen peroxide determination*
167 The concentration of hydrogen peroxide was determined by measuring the change
168 in absorbance of the reaction mixture at 500 nm. A total volume of 1 ml of the

reaction mixture consisted of 0.17 mM *o*-dianisidine-HCl (Sigma) in 20 mM PB (24.4 mM Na_2HPO_4 and 15.6 mM NaH_2PO_4, pH 7.0), 60 U/ml horseradish peroxidase (Sigma), and 34.5 μl of a sample taken from the culture medium. For the negative control, 34.5 μl of 20 mM PB was added instead of the sample. The samples were added and incubated at 35°C for 5 min, and then, the absorbance at 500 nm was recorded.

3. Results

3. 1. ASG culture in extra volumes of Grace's medium

During the culture of V3 ASGs in Grace's medium, the ASGs generated GOD (Matsui et al., 2011). The GOD utilized glucose in the medium as a substrate to produce H_2O_2. This H_2O_2 inhibited V3 ASGs from responding to 20E with PCD. This inhibitory effect made it difficult to determine the time at which ASGs became competent to respond to 20E. V3 ASGs cultured in Ringer's solution died due to a lack of glucose in the solution; thus, no H_2O_2 was generated by these ASGs. The V3 ASGs survived when the Ringer's solution was supplemented with 0.01% H_2O_2 (approximately 3 mM) but died at 0.005% H_2O_2. These results indicate that a minimum concentration of H_2O_2 in the medium was crucial to inhibit the action of 20E. Thus, the ASGs were cultured in various volumes of the medium to reduce the GOD concentration and thereby reduce the H_2O_2 concentration to reduce its inhibitory effects. We cultured V3 ASGs in the medium at volumes equal to 10, 20 or 30 times higher than the standard culture volume of 0.3 ml (Fig. 1). V3 ASGs survived when cultured in 0.3 or 3 ml of medium. In 6 ml, however, the ASGs showed some but not all of the morphological changes

associated with PCD. In 9 ml of medium, the ASGs underwent cell death with a PCD score of 4.8 ± 1.1 (Fig. 1A). These values indicated that the V3 ASGs were sensitive to 20E, but the GOD/H_2O_2 may have suppressed the progress of PCD.

To test this hypothesis, we measured the changes in H_2O_2 concentration during culture in 0.3, 3, 6 or 9 ml of medium (Fig. 1B-E). When an ASG was cultured in 9 ml of medium, the H_2O_2 concentration at 72 h was 0.31 ± 0.071 mM. In contrast, during culture in 0.3 ml of medium, the H_2O_2 concentration was 0.625 ± 0.107 mM at 3 h (Fig. 1B). Accordingly, an increase in the medium volume resulted in the death of the V3 ASGs. The death of the ASGs was a result of the reduction of the H_2O_2 concentration below an inhibitory level. Therefore, we used 9 ml of the medium in the experiments described below.

3.2. The timing of the acquisition of 20E responsiveness

The time when ASGs become responsive to 20E was determined by culturing the ASGs every day from IV4 to V7 in 9 ml of medium with 20E for 144 h. The majority of IV4, V0 and V1 ASGs exhibited the type B form (Fig. 4A; see Kakei et al., 2005 for type B form). Specifically, the cells exhibited blebbing, the nuclear morphology at 144 h showed small amounts of cell and nuclear condensation (Fig. 2D, d). The PCD scores of the others were below 3. In contrast, when the V2 ASGs were cultured in 9 ml of the medium with 20E, no type B forms were observed. Instead, approximately 40% of the ASGs completed PCD (Fig. 2F, f') and had PCD scores of 6. Most of the remaining ASGs attained PCD scores of 5 (average PCD score, 4.9 ± 1.0; Fig. 2A). In these ASG cells, DNA fragmentation occurred (Fig. 2B, f'), but not in the V1 cells (Fig. 2B). These results indicate that the V2 ASGs were

competent to respond to 20E by completing PCD.

To examine whether the acquisition of responsiveness to 20E is associated with specific gene expression changes, the expression of early and early-late genes was determined for V1 and V2 ASGs (Fig. 2G). Among the genes examined, the expression levels of five genes, *EcR-B1* (ecdysone receptor-B1), *E75A*, *E75B*, *βFTZ-F1* and *BHR3* (*Bombyx* hormone receptor 3), were equally enhanced in response to 20E in both ASGs. This result indicates that those genes may not be involved in the change in the responsiveness. Among the 13 genes examined, only the level of *E74B* exhibited a difference between the V1 and V2 ASGs in the absence as well as in the presence of 20E. The *E74B* basal expression (in the absence of 20E) was almost undetectable on V1 but became detectable on V2. Moreover, 20E greatly enhanced the expression of *E74B* in V2 ASGs, but only slightly increased its expression in V1 ASGs. Among the three *BR-C* isoforms, only the Z1 isoform was expressed in those ASGs. 20E exerted no effects on Z1 isoform mRNA expression in the V1 ASGs but increased it in the V2 ASGs. However, the basal expression level of the Z1 isoform decreased from V1 to V2, indicating that the decrease could be important to the acquisition of competence to respond to 20E.

3.3. Suppression of the change in the responsiveness to 20E

The results above show that V2 ASGs are competent to respond to 20E by undergoing cell death. If this early phenomenon were associated with the change in commitment to die, JH should affect the responsiveness to 20E. To investigate this issue, CA were removed from IV3 larvae, and JHA or acetone was topically

applied immediately after the removal. The ASGs of the larvae that successfully molted into the fifth instar were cultured in 9 ml of the medium with 20E on V0 and V3. The ASGs of allatectomized V0 larvae completed PCD in 83% of the glands, while those that were treated with JHA did not undergo PCD. Specifically, the PCD score was 3 in approximately 30% of the JHA-treated glands, and the remaining glands exhibited the type B morphology that showed no nuclear fragmentation (Fig. 3A), which was similar to the response to 20E of the intact V1 larval ASGs. Similar results were obtained for the ASGs that were examined on V3 (Fig. 3B), although the intact V3 larval ASGs underwent PCD under the same culture conditions. These results indicate that JHA is involved in the acquisition of responsiveness to 20E.

3.4. The timing of the loss of sensitivity to JH

To determine the time when ASGs lose their sensitivity to JH, fifth instar larvae were treated with JHA, and the responsiveness of ASGs of those larvae were examined in vitro two days later (Fig. 4A). The gland response to 20E was suppressed when JHA was applied on or before V4. In those ASGs, 40-90% of the glands exhibited the type B form, and the remaining glands had PCD scores below 3. In larvae treated with JHA on V5, approximately half of the ASGs underwent PCD (PCD scores of 5 or 6), but the other half of the ASGs exhibited responses similar to the glands of larvae treated with JHA before V5. V6 ASGs exhibited a complete response to 20E, which demonstrates that the ASGs began to lose sensitivity to JH around V5 and completely lost it between V5 and V6.

To examine the time when the ASGs become insensitive to JHA, we applied

JHA on each day of the fifth instar, and examined the gland responses to 20E in vitro on V8.. Figure 5 shows that the JHA applied on V2-V5 was effective at preventing most of the 20E-induced cell death in the glands. The inhibitory effect of applying JHA on V2 lasted until V8, at which time the gland's response was examined. When given JHA on V5 and assayed on V8, 42% exibited the Type B form and the remaining ASGs scored 2-3 in PCD, whereas in the larvae allatectomized on IV3, the ASGs treated with JHA at the same time and assayed two days later showed more extensive PCD (Fig. 4A). These results indicate that the V5 ASGs were less responsive to 20E than those shown in Fig. 4.

3.5. Kr-h1 *expression and JHA*

In the intracellular signaling pathway of JH, methoprene-tolerant protein (Met) acts as a JH receptor, and Krüppel homolog 1 (Kr-h1) plays a key role in the hierarchical control of the expression of Met downstream target genes (Minakuchi et al., 2008; Konopova et al., 2011). To examine whether the loss of sensitivity to JH is associated with *Kr-h1* expression, we measured *Kr-h1* expression by RT-PCR. In intact larval ASGs, *Kr-h1* expression was high on V0, low but detectable on V1, and very low on V2 (Fig. 3C, left panel). V0 larvae were topically treated with JHA or acetone, and their ASGs were examined on V3. The *Kr-h1* expression in the acetone-treated ASGs was very low on V3 (similar to intact larval ASGs), while in the JHA treatment group, the expression level was as high as in V0 ASGs. Accordingly, *Kr-h1* expression appeared to be retained in the presence of JH (Fig. 3C, right panel) irrespective of the larval age.

JHA treatment enhanced *Kr-h1* expression irrespective of the acquisition of responsiveness to 20E (Fig. 4). Larvae were allatectomized on IV3, and JHA was

applied on V4, 5 or 6. The expression level was examined 2 days later (Fig. 4B). A single application of JHA greatly increased *Kr-h1* expression irrespective of the day of JHA application, showing that *Kr-h1* expression depends on the presence of JHA.

4. Discussion

A series of changes in the tissue responses to 20E and JH is an indication of the change in commitment (Riddiford, 1985); the acquisition of responsiveness to 20E precedes to the loss of sensitivity to JH (Riddiford, 1985 for epidermis, Obara et al., 2002 for wing discs). Tissues such as epidermis and imaginal discs are pupally committed during feeding period of the last-larval instar and undergo morphogenetic changes to form pupal tissues during pupal metamorphosis. In contrast, it remained unclear whether larval-specific tissues that are eliminated from insect bodies are destined to die and whether there is a death commitment occurring in the larval period. Using *B. mori* ASGs, we were able to partially answer these questions.

The *B. mori* ASGs became responsive to 20E by undergoing PCD on V2, and they lost sensitivity to JH on V6 (the end of the feeding period) (Fig. 6). The ASGs progress to death through a series of steps. 20E did not induce PCD in V1 ASGs even when they were cultured in 9 ml of medium, but V2 ASGs underwent PCD when cultured under the same conditions. This result clearly shows that ASGs became responsive to 20E between V1 and V2. The acquisition of responsiveness required a disappearance of JH from the larvae. When the CA were removed on IV3, the ASGs of V0 larvae underwent PCD, while the V0 intact larval ASGs

never did so. Alternatively, topical application of JHA to intact larvae on V0 suppressed the acquisition of responsiveness to 20E. These results indicate that maintaining JH in ASGs led to inhibition of responsiveness to 20E.

Our previous study suggested that the sensitivity to JH may be lost between V4 and V5 (Kakei et al., 2005). The present study clarified the timing of the loss of sensitivity. JHA application on V4 prevented the ASGs from undergoing PCD by 20E, but the application on V5 failed to inhibit the 20E response in one-third of the ASGs examined. In addition, JHA applied on V5 did not inhibit the 20E-induced PCD to any degree. Thus, the ASGs may begin to lose their sensitivity between V4 and V5 and lose it entirely by V6. Because it is recognized that the pupal commitment is completed when a given tissue loses its sensitivity to JH (Riddiford, 1985), the death commitment in ASGs may be completed on V6.

Prior to V6, ASGs do not undergo PCD under standard culture conditions (Matsui et al., 2011). The concentration of JH in the hemolymph is very low between V2 (the day of acquisition of responsiveness to 20E) and V6 (the loss of sensitivity to JH) (Sakurai and Niimi, 1997), indicating that JH may not be involved in the lack of response to 20E during this period. GOD/H_2O_2 in ASGs inhibits the gland response to 20E (Matsui et al., 2011), and in the ASGs between V2 and V5, GOD may play a critical role in inhibiting PCD. The critical concentration of 20E to induce PCD is 1 μM (Terashima et al., 2000). Although this concentration has not been recorded during the feeding period of any lepidopteran larvae (Dean et al., 1980; Koyama et al., 2004), GOD may serve as a biochemical assurance that ASGs never undergo PCD in response to an unexpected rise in hemolymph ecdysteroid levels during the feeding period. GOD

337 in the ASG inner cavity is discarded with released silk proteins in the early phase
338 of cocooning. After discarding GOD, the ASGs are allowed to respond to 20E.
339 Consequently, they are eliminated through PCD, which is triggered by a
340 metamorphic rise in hemolymph ecdysteroid concentration. Thus, the
341 20E-induced PCD of ASGs is controlled by a stage-specific dual regulation.

342 The dual regulation in *B. mori* ASGs is unique among animal tissues that are
343 eliminated at metamorphosis. In amphibians, most tissues of the tadpole undergo
344 a complete transformation from larval to adult stage. Thyroid hormone (TH) plays
345 a critical role in the degeneration of larval tissues and the concurrent
346 proliferation and differentiation of adult tissues in a developmental
347 stage-dependent manner. The elimination of the larval tail is largely achieved
348 through cell apoptosis during the climax (NF stage 58-65), at which point TH
349 levels are at their maximum. Tails isolated from tadpoles as late as stage 49 do
350 not respond to high concentrations of TH, while those from stage 51 can be
351 induced to undergo resorption (Shaffer, 1963). At early stages of metamorphosis,
352 the larval tails express low levels of the gene encoding the TH-inactivating
353 enzyme type III deiodinase (D3) (Kawahara et al., 1999). At the onset of tail
354 resorption, *D3* expression declines, and the gene encoding type II deiodinase (D2)
355 is upregulated. D2 catalyzes the deiodination of the outer ring of T4, which results
356 in a more potent thyroid hormone, T3 (Cai and Brown, 2004). In tails, *TRα* and
357 *TRβ* mRNA levels rise in parallel with an increase in endogenous TH
358 concentration (Wang and Brown, 1993), and adequate receptor expression is
359 necessary to obtain tissue sensitivity to TH (Wang and Brown, 1993). Thus, the
360 timing of death in amphibian tails may be regulated primarily by TH and

361 secondarily by the expression of other genes, such as *D2*, *D3* and *TR*, which may
362 be influenced by the changing TH concentrations.
363 In ASGs, the expression levels of EcR and EcR partner protein, Ultraspiracle
364 (Usp), do not change remarkably from V1 to V2 (Kaneko et al., 2006). This lack of
365 change indicates a lack of relationship between *EcR* expression and the
366 acquisition of responsiveness to 20E, in contrast to the dynamic of TH receptors in
367 amphibian tails. Culturing V2 ASGs in 9 ml of the medium with 20E induced
368 DNA fragmentation, but this was not the case for V1 ASGs. This result indicates
369 that the 20E signaling pathway must be completed to the point of activation of
370 caspase 3-like protease (Iga et al., 2010) in V2 ASGs but not in V1 glands.
371 Accordingly, some factor(s) in the pathway must be up- or down-regulated
372 between V1 and V2, but we could not find any early genes with notable differences
373 in expression between V1 and V2 ASGs.
374 JH application before V5 inhibited the execution of PCD; the PCD scores
375 remained less than 4, but type B forms appeared in 40-90% of specimens. The type
376 B form is characterized by the appearance of plasma membrane blebbing, cell
377 shrinkage and nuclear condensation, but nuclear and DNA fragmentation do not
378 occur in these cells (Fig. 2; Kakei et al., 2005). Nuclear and DNA fragmentation
379 are under the control of the PKC-caspase 3 pathway (Iga et al., 2010), while the
380 pathway up to nuclear condensation is likely mediated by the CaMK pathway
381 (Manaboon, 2009) and cell shrinkage is under the genomic pathway (Iga et al.,
382 2007). Accordingly, the present results indicate that JH may inhibit the
383 non-genomic pathway up to caspase 3-like protease activation leading to DNA and
384 nuclear fragmentation. The final process of apoptosis, which is associated with

385 nuclear and DNA fragmentation by activated caspase 3-like protease, is under the
386 developmental regulation of the death commitment occurring on V6. Since the
387 type B form occurs in ASGs before V2, ASGs are competent to respond to 20E in
388 vitro by exhibiting nuclear condensation and cell shrinkage. These phenomena do
389 not occur in vivo at the end of the fourth instar, when ASGs experience a peak
390 hemolymph ecdysteroid concentration of approximately 1 μM (Koyama et al.,
391 2004), and the mechanism of inhibition of apoptosis at the time of larval-larval
392 molting remains unknown.

393 JH controls gene expression through its putative receptor Met and Kr-h1 that
394 is an early JH-response gene located downstream of Met (Minakuchi et al., 2008,
395 2009; Liu et al., 2009; Konopova et al., 2011; Lozano and Belles, 2011). In the *D.*
396 *melanogaster* fat body, JH counteracts Met to prevent caspase-dependent PCD
397 and thereby controls fat body remodeling and larval-pupal metamorphosis. In *B.*
398 *mori*, *Kr-h1* expression declined to very low level on V2, when the in vivo JH titer
399 is very low (Sakurai and Niimi, 1997). A single application of JHA, however,
400 enhanced *Kr-h1* expression irrespective of the time of JH application. This effect
401 was observed even on V6, when the ASGs were committed to die and not
402 responsive to JHA, indicating that Kr-h1 is not involved in the change in
403 commitment. Also, Kr-h1 may not mediate the JHA counteraction to inhibit
404 20E-induced DNA fragmentation, because JHA application on V6 did not inhibit
405 the 20E-induced PCD, while inducing high *Kr-h1* expression (present results,
406 Kakei et al., 2005). Moreover, these observations suggest that, in this case, JH
407 action is mediated by an alternative signaling pathway that does not use Kr-h1 as
408 a transducer.

409

410 ## Acknowledgments

411 This work was supported by the JSPS Grant-in-Aid for Scientific Research
412 21380035 (to S.S.).

413

References

Cai, L., Brown, D.D., 2004. Expression of type II iodothyronine deiodinase marks the time that a tissue responds to thyroid hormone-induced metamorphosis in *Xenopus* laevis. Developmental Biology 266, 87-95.

Chinzei, Y., 1975. Induction of histolysis by ecdysterone *in vitro*: breakdown of anterior silk gland in silkworm *Bombyx mori* (Lepidoptera: Bombycidae). Applied entomology and zoology 10, 136-138.

Chomcyznski, P., Sacchi, N., 1987. Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Analytical Biochemistry 162: 156-159.

Dean, R.L., Bollenbacher, W.E., Locke, M., Smith, S.L., Gilbert, L.I., 1980. Haemolymph ecdysteroid levels and cellular events in the intermoult/moult sequence of *Calpodes ethlius*. Journal of Insect Physiology 26, 267-280.

Iga, M., Manaboon, M., Matsui, H., Sakurai, S., 2010. Ca^{2+}-PKC-caspase 3-like protease pathway mediates DNA and nuclear fragmentation in ecdysteroid-induced programmed cell death. Molecular and Cellular Endocrinology 321, 146-151.

Kakei, M., Iwami, M., Sakurai, S., 2005. Death commitment in the anterior silk gland of the silkworm, *Bombyx mori*. Journal of Insect Physiology 51, 17-25.

Kawahara, A., Gohda, Y., Hikosaka, A., 1999. Role of type Ⅲ iodothyronine 5-deiodinase gene expression in temporal regulation of *Xenopus* metamorphosis. Development, growth & differentiation 41, 365-373.

Kaneko, Y., Takaki, K., Iwami, M., Sakurai, S., 2006. Developmental profile of annexin IX and its possible role in programmed cell death of the *Bombyx mori*

438 anterior silk gland. Zoological Science 23, 533-542.
439 Konopova, B., Smykal, V., Jindra, M., 2011. Common and distinct roles of juvenile
440 hormone signaling genes in metamorphosis of holometabolous and
441 hemimetabolous insects. PloS One 6, e28728.
442 Koyama, T., Obara, Y., Iwami, M., Sakurai, S., 2004. Commencement of pual
443 commitment in late penultimate instar and its hormonal control in wing
444 imaginal discs of the silkworm, *Bombyx mori*. Journal of Insect Physiology 50,
445 123-133.
446 Liu, Y., Sheng, Z., Liu, H., Wen, D., He, Q., Wang, S., Shao, W., Jiang, R-J., An, S.,
447 Sun, Y., Bendena, W. G., Wang, J., Gilbert, L. I., Wilson, T. G., Song, Q., Li, S.,
448 2009. Juvenile hormone counteracts the bHLH-PAS transcription factors MET
449 and GCE to prevent caspase-dependent programmed cell death in *Drosophila*.
450 Development 136, 2015-2025.
451 Lozano, J. and Belles, X., 2011. Conserved repressive function of Krüppel homolog
452 1 on insect metamorphosis in hemimetabolous and holometabolous species.
453 Scientific Reports 1, 163.
454 Manaboon, M., Iga, M., Iwami, M., Sakurai, S., 2009. Intracellular mobilization of
455 Ca^{2+} by the insect steroid hormone 20-hydroxyecdysone during programmed cell
456 death in silkworm anterior silk glands. Journal of Insect Physiology 55, 122-128.
457 Matsui, H., Kakei, M., Iwami, M., Sakurai, S., 2011. Glucose oxidase prevents
458 programmed cell death of the silkworm anterior silk gland through hydrogen
459 peroxide production. FEBS Journal 278, 776-785.
460 Minakuchi, C., Zhu, X., Riddiford, L. M., 2008. *Krüppel homolog 1* (*Kr-h1*)
461 mediates juvenile hormone action during metamorphosis of *Drosophila*

462 *melanogaster*, Mechanism Development 125, 91-105.
463 Minakuchi, C., Namiki, T., Shinoda, T., 2009. *Krüppel homolog 1*, an early
464 juvenile hormone-response gene downstream of *Methoprene-tolerant* mediates
465 its anti-metamorphic action in the red flour beetle *Tribolium castaneum.*
466 Developmental Biology 325, 341-350.
467 Obara, Y., Miyatani, M., Ishiguro, Y., Hirota, K., Koyama, T., Izumi, S., Iwami, M.,
468 Sakurai, S., 2002. Pupal commitment and its hormonal control in wing imaginal
469 discs. Journal of Insect Physiology 48, 933-944.
470 Riddiford, L. M., 1985. Hormone action at the cellular level. In: Kerkut, G. A.,
471 Gilbert, L. I. (Eds), Comprehensive insect physiology, biochemistry, and
472 pharmacology, Vol. 8. Pergamon Press, New York, pp. 37-84.
473 Sakurai, S., 1983. Temporal organization of endocrine events underlying
474 larval-pupal metamorphosis in the silkworm, *Bombyx mori*. Journal of Insect
475 Physiology 29, 919-932.
476 Sakurai, S., Kaya, M., Satake, S., 1998. Hemolymph ecdysteroid titer and
477 ecdysteroid-dependent developmental events in the last-larval stadium of the
478 silkworm, *Bombyx mori*: role of low ecdysteroid titer in larval pupal
479 metamorphosis and a reappraisal of the head critical period. Journal of Insect
480 Physiology 44, 867-881.
481 Sakurai, S., Niimi, S., 1997. Developmental changes in juvenile hormone and
482 juvenile hormone acid titers in the hemolymph and in vitro juvenile hormone
483 synthesis by corpora allata of the silkworm, *Bombyx mori*. Journal of Insect
484 Physiology 43, 875-884.
485 Schwartz, L. M., 1992. Insect muscle as a model for programmed cell death.

Journal of Neurobiology 23, 1312-1326.

Sekimoto, T., Iwami, M., Sakurai, S., 2006. Coordinate responses of transcription factors to ecdysone during programmed cell death in the anterior silk gland of the silkworm, *Bombyx mori*. Insect Molecular Biology 15, 281-292.

Sekimoto, T., Iwami, M., Sakurai, S., 2007. 20-Hydroxyecdysone regulation of two isoforms of the Ets transcription factor E74 gene in programmed cell death in the silkworm anterior silk gland. Insect Molecular Biology 16, 581-590.

Shaffer, B. M., 1963. The isolated *Xenopus* laevis tail: a preparation for studying the central nervous system and metamorphosis in culture. Journal of embryology and experimental morphology 11, 77-90.

Terashima, J., Yasuhara, N., Iwami, M., Sakurai, S., 2000. Programmed cell death triggered by insect steroid hormone, 20-hydroxyecdysone, in the anterior silk gland of the silkworm, *Bombyx mori*. Development Genes and Evolution 210, 545-558.

von Gaudecker, B., Schmale, E. M., 1974. Substrate-histochemical investigations and ultrahistochemocal demonstrations of acid phosphatase in larval and prepupal salivary glands of *Drosophila melanogaster*. Cell and Tissue Research 155, 75-89.

Wang, Z., Brown, D. D., 1993. Thyroid hormone-induced gene expression program for amphibian tail resorption. The Journal of Biological chemistry 268, 16270-16278.

Figure Legends

Figure 1. V3 ASGs underwent PCD in an excess volume of Grace's medium. V3 ASGs were cultured individually in 0.3, 3, 6 or 9 ml of medium with 1 μM 20E for 144 h. (A) The degree of PCD progression was noted with PCD scores as described by Terashima et al. (2000). A score of 0 indicates no change, and a score of 6 indicates the completion of PCD. The PCD score was noted every 24 h. (B-E) The concentration of H_2O_2 in medium was measured during the culture of the V3 ASGs in 0.3-9 ml of medium. Each point represents the mean ± SD; n=12 for (A) and 3 for (B-E).

Figure 2. ASGs become competent to respond to 20E on V2. (A) ASGs from IV4 to V7 were incubated in 9 ml of Grace's medium with 1 μM 20E (+) or without 20E (-) for 144 h. The ordinate indicates the percentage of the glands that exhibited a given PCD score at the end of the culture. ASGs that scored at or above 4 underwent PCD (Terashima et al., 2000; Iga et al., 2007). Some of the ASGs exhibited the type B form (Kakei et al., 2005), which is indicated with a "B" in the PCD score (n=12 for each column). (B) The oligonucleosomal ladder first appeared in V2 ASGs when cultured with 20E (V2 +20E). The left column shows a 100-bp DNA fragment ladder. In V1 ASGs, the DNA appears as a smear. (C, c) V1 ASGs after 144 h of culture in Grace's medium without 20E or (D, d) with 20E. (E, e) V2 ASGs after 144 h of culture in the medium without 20E or (F, f) with 20E. (C-F) Light micrograph and (c-f) DAPI staining show nuclear morphology. Panels (C,E,c,e) and (F,f) show typical examples of score 0 and score 6, respectively. Panels (D,d) are typical examples of the type B form. Scale bar, 70 μm. (G)

532 Expression of 20E-responsive genes in V1 and V2 ASGs. ASGs were incubated in
533 9 ml of the medium with 20E for 8 h. The genes *EcR*, ecdysone receptor; *usp*,
534 Ultraspiracle; *BR-C*, broad complex; *BHR3*, *Bombyx* hormone receptor 3; and
535 *RpL3*, ribosomal protein L3 were selected according to Sekimoto et al. (2006;
536 2007).
537
538 **Figure 3.** Change in the timing of the death commitment in ASGs. (A) A pair of
539 CA were removed from individual larvae on day 3 of the fourth instar (IV3).
540 Acetone or 0.1 μg of JHA was topically applied within hours after the operation.
541 The ASGs of the larvae that successfully molted into the fifth instar were cultured
542 on the same day in 9 ml of Grace's medium with 1 μM 20E. (B) Larvae were
543 allatectomized on IV3, and the newly molted V0 larvae were treated with acetone
544 or 0.1 μg of JHA. ASGs were obtained on V3 and cultured in 9 ml of the medium
545 with 20E for 144 h. Ordinates in (A, B) are the same as in Figure 2A (n=12 for
546 each column in (A) and (B)). (C) Expression of the *Kr-h1* gene in V0, V1 and V2
547 ASGs is shown in intact larvae (left panel) and in ASGs of the larvae that received
548 the same treatments as those in (B) (right panel).
549
550 **Figure 4.** Involvement of JH in the acquisition of responsiveness to 20E. The CA
551 were removed on IV3, and those larvae received a single application of JHA (0.1
552 μg/larva) on a given day between V0 and V6. ASGs were obtained two days after
553 JHA application and were cultured in 9 ml of medium with 20E for 144 h. (A)
554 Responses to 20E as noted with PCD scores (see Fig. 2 for scores) (n=10 for V0-3
555 and V6; 18 for V4; and 12 for V5. (B) *Kr-h1* expression in ASGs at the time of

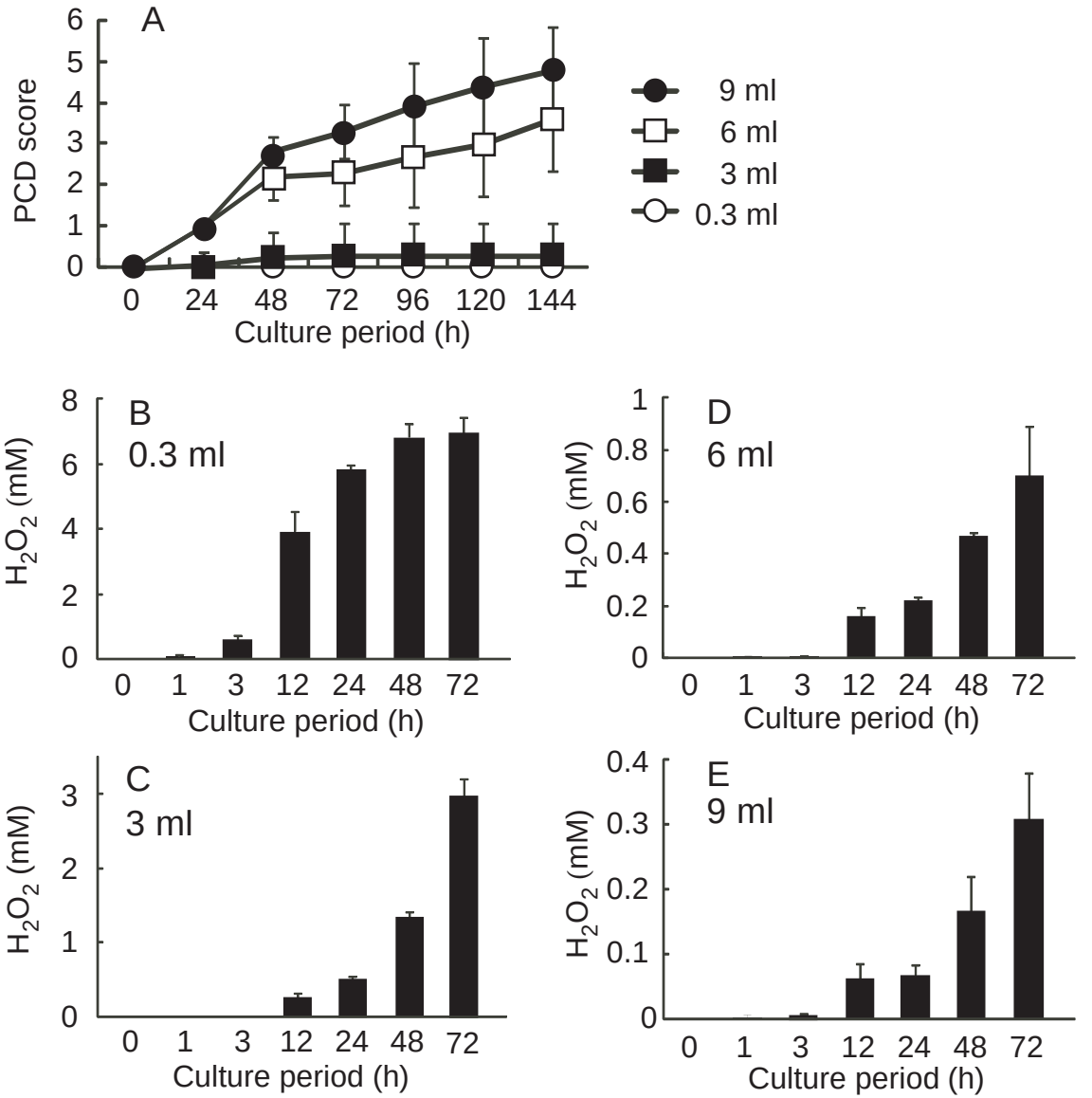
556 dissection.

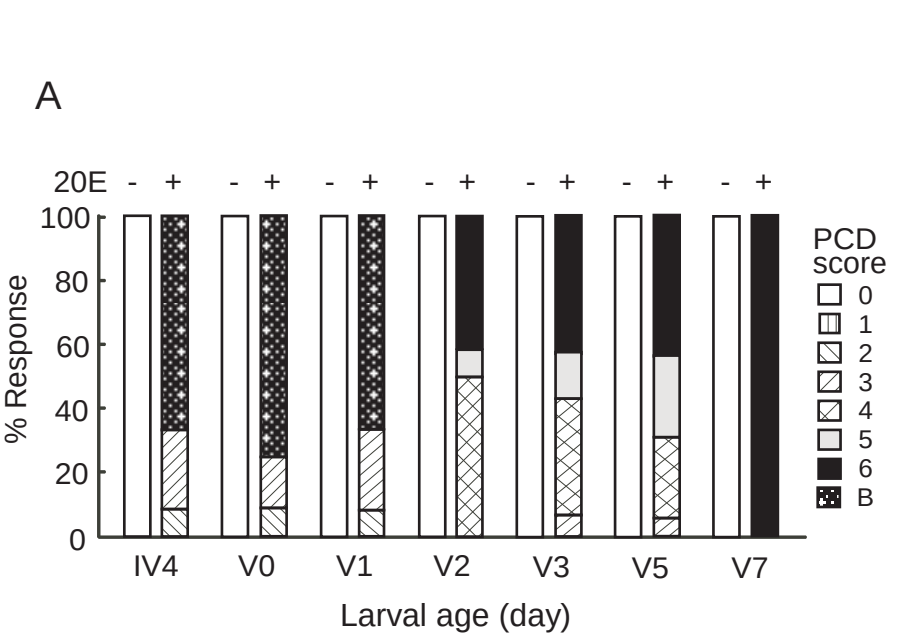
557

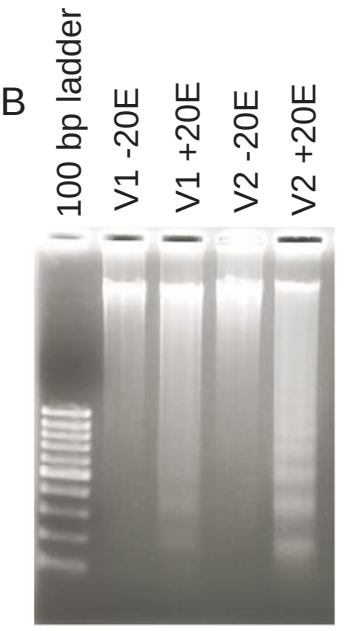
558 **Figure 5.** ASGs begin to lose their sensitivity to JHA on V5 and completely lose
559 it on V6. Fifth instar larvae were topically treated with acetone (-) or a single dose
560 of JHA (0.1 μg/larva) (+) on the day indicated. ASGs were dissected on V8
561 irrespective of the day of treatment and cultured in 9 ml of medium with 20E for
562 144 h. The ordinate is the same as in Figure 2 (n=12 for each column).

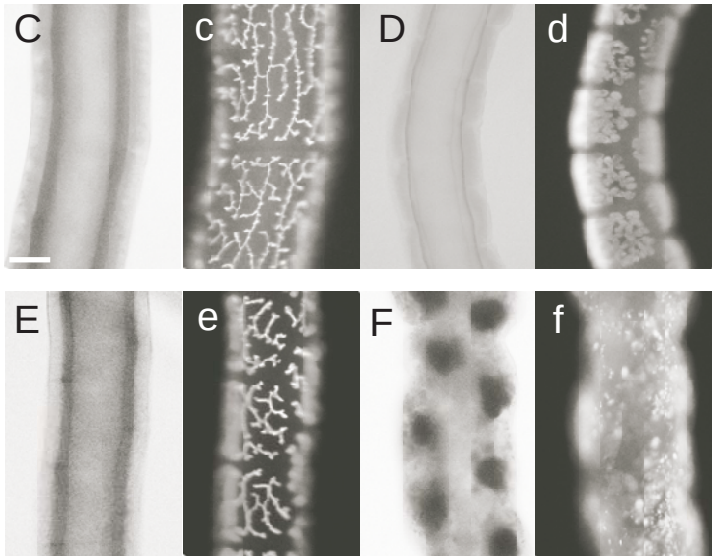
563

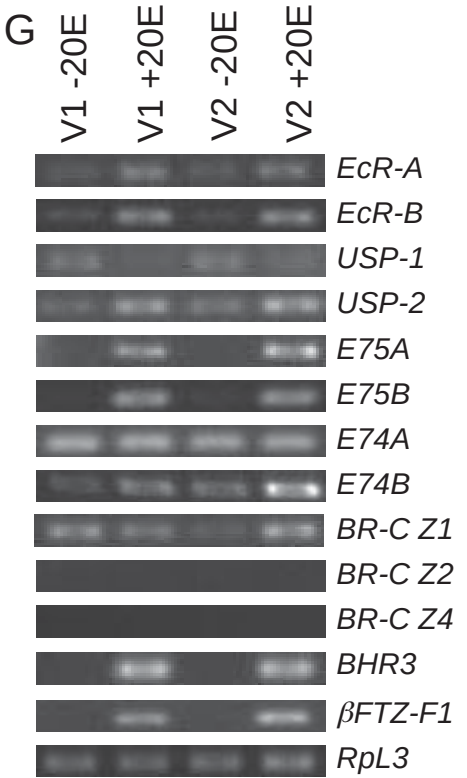
564 **Figure 6.** Schematic representation of a proposed hormonal regulation of the
565 death commitment and the progression of PCD execution in *B. mori* ASGs. The
566 solid and broken lines indicate ecdysteroids and JH concentration in the
567 hemolymph, respectively (Satake et al., 1998; Sakurai and Niimi, 1997).

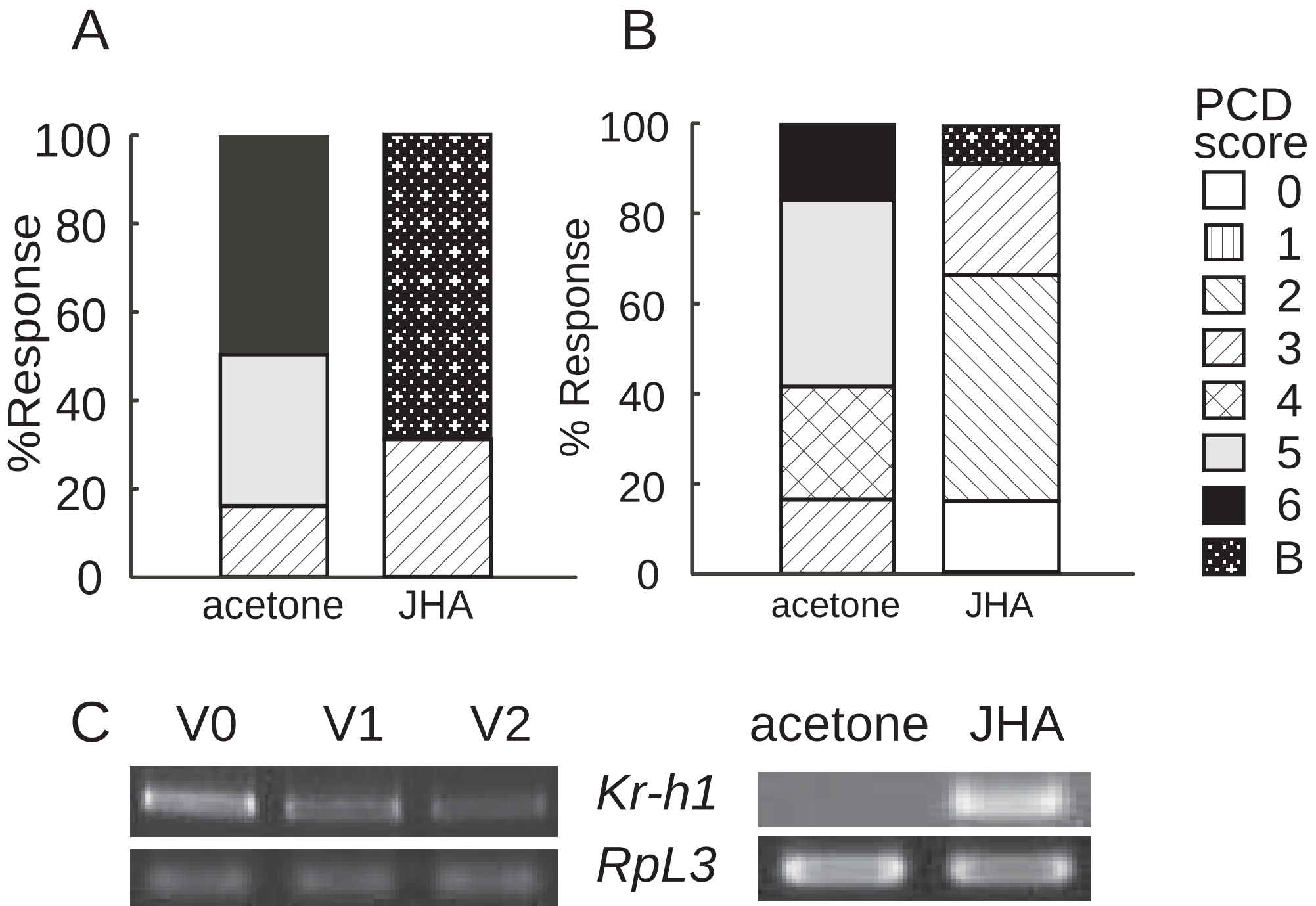

A
PCD score
0
1
2
3
4
5
6
0 24 48 72 96 120 144
Culture period (h)
9 ml
6 ml
3 ml
0.3 ml
B
0.3 ml
H_2O_2 (mM)
0
2
4
6
8
0 1 3 12 24 48 72
Culture period (h)
D
6 ml
H_2O_2 (mM)
0
0.2
0.4
0.6
0.8
1
0 1 3 12 24 48 72
Culture period (h)
C
3 ml
H_2O_2 (mM)
0
1
2
3
0 1 3 12 24 48 72
Culture period (h)
E
9 ml
H_2O_2 (mM)
0
0.1
0.2
0.3
0.4
0 1 3 12 24 48 72
Culture period (h)

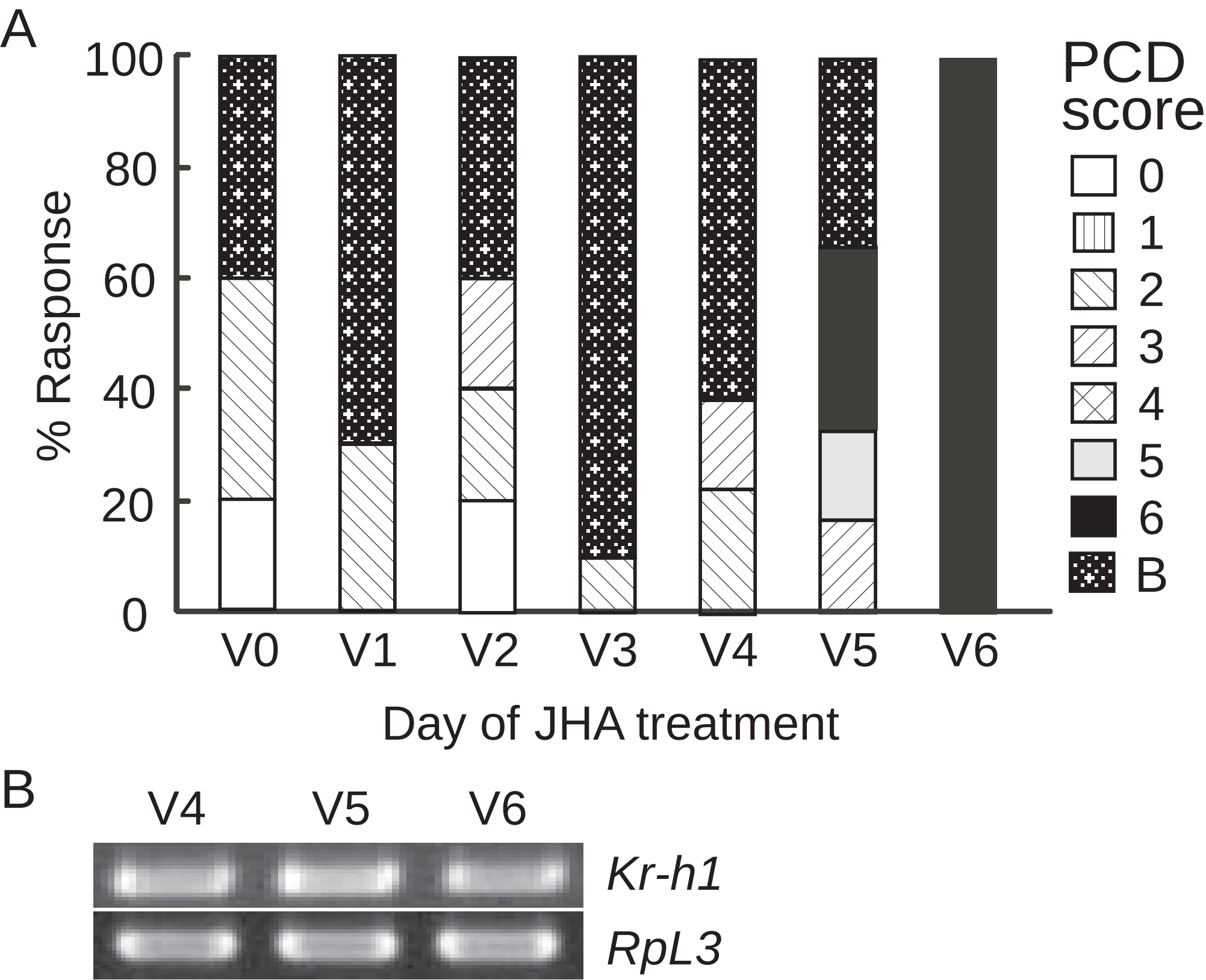

A
20E - + - + - + - + - + - + - +
100
80
60
40
20
0
% Response
IV4 V0 V1 V2 V3 V5 V7
Larval age (day)
PCD score
0
1
2
3
4
5
6
B

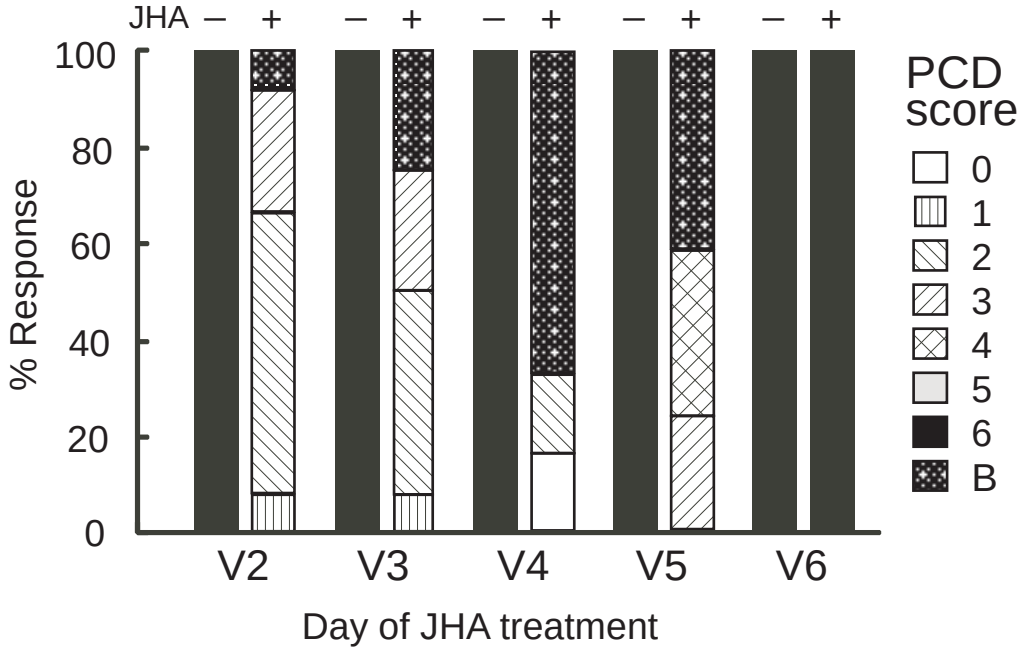

B
100 bp ladder
V1 -20E
V1 +20E
V2 -20E
V2 +20E

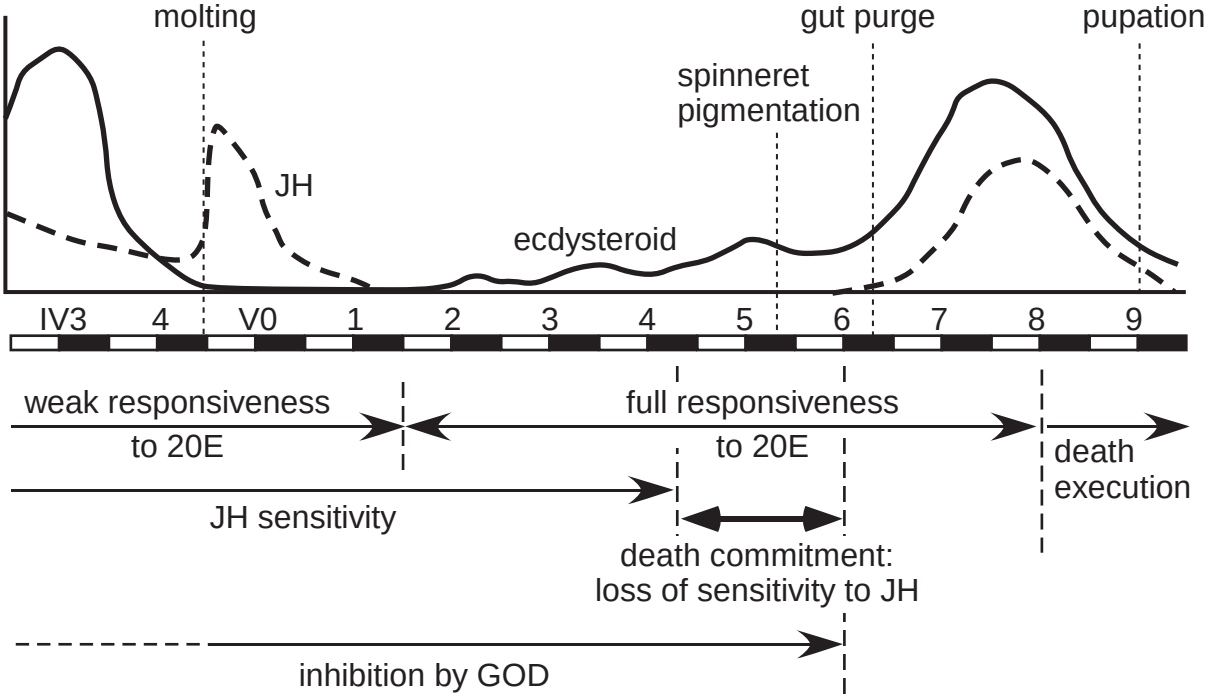

C
c
D
d
E
e
F
f


G
V1 -20E
V1 +20E
V2 -20E
V2 +20E
EcR-A
EcR-B
USP-1
USP-2
E75A
E75B
E74A
E74B
BR-C Z1
BR-C Z2
BR-C Z4
BHR3
βFTZ-F1
RpL3


A
B
100
80
60
40
20
0
%Response
% Response
acetone
JHA
PCD score
0
1
2
3
4
5
6
B
C
V0
V1
V2
acetone
JHA
Kr-h1
RpL3


A
100
80
60
40
20
0
% Rasponse
V0
V1
V2
V3
V4
V5
V6
Day of JHA treatment
PCD score
0
1
2
3
4
5
6
B
B
V4
V5
V6
Kr-h1
RpL3


JHA – + – + – + – + – +
100
80
60
40
20
0
% Response
V2
V3
V4
V5
V6
Day of JHA treatment
PCD score
0
1
2
3
4
5
6
B


molting
gut purge
pupation
spinneret
pigmentation
JH
ecdysteroid
IV3
4
V0
1
2
3
4
5
6
7
8
9
weak responsiveness
to 20E
full responsiveness
to 20E
death
execution
JH sensitivity
death commitment:
loss of sensitivity to JH
inhibition by GOD