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# Juvenilizing effect of ecdysone mimic RH 5849 in *Galleria mellonella* larvae [1]

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*Abstract.* The response of the final instar larvae of *G. mellonella* to topical application of the non-steroidal ecdysone mimic, RH 5849, was age-related as well as dose-dependent. In young final instar larvae, moderate doses of RH 5849 induced perfect supernumerary larval moults, but doses equal to and higher than 8.5 µg per larva caused premature formation of larval cuticle and were lethal. Application of RH 5849 significantly increased allatotropic activity of the brain, and also activated synthesis of juvenile hormone (JH) by the corpora cardiaca/corpora allata complex. Simultaneous application of RH 5849 and FMev, a potent inhibitor of JH synthesis, to young final instar larvae lowered the incidence of perfect supernumerary larval moults. We conclude that the effect of RH 5849 on the developmental programme in *G. mellonella* is mediated by the corpora allata.
*Key words. Galleria mellonella*; ecdysone mimic RH 5849; juvenile hormone; allatotropic activity; corpora allata; supernumerary larval moult; juvenilizing effect.

It is generally accepted that in lepidopteran species a drop in juvenile hormone (JH) titre at the beginning of the final larval instar facilitates the metamorphic developmental programme, which is then controlled by two ecdysteroid peaks. A small peak precedes onset of wandering and a larger peak occurs just before pupation [2–6]. In *G. mellonella* the metamorphic programme is labile and the larva can revert to the larval programme under the influence of chilling stress [7], brain implantation [8] or JH application [9]. This shift is externally manifested by a supernumerary larval moult or a moult to the larval-pupal intermediate [9, 10]. The larval programme is characterized by a high JH titre throughout instar and a peak of ecdysteroids which reaches about 300 ng per g of body weight in *G. mellonella* [2, 11]. According to Sehnal et al. [2] the shape and size of the ecdysteroid peak during the larval cycle is regulated by levels of JH. Following bilateral allatectomy or application of FMev, an inhibitor of JH synthesis [12], larvae lose the ability to produce supernumerary larvae [8, 13, 14]. It is claimed that high levels of ecdysteroids mimic the action of JH, possibly by activating the insect's own corpora allata, or by acting synergistically with low levels of endogenous JH (for review see Willis [14]). However, the rapid degradation of ecdysteroids in insect tissues has been a serious obstacle in these studies. Recently, a highly potent, metabolically stable, non-steroidal ecdysone mimic, RH 5849, has been discovered [16]. This compound causes premature initiation of the larval moult at all stages of larval development of *Manduca sexta* [17] and in other insect species [18]. In larvae of *Plodia interpunctella*, RH 5849 stimulates supernumerary larval moults only when applied together with methoprene [19]. Application of RH 5849 performed very early in the final larval instar of diapausing and non-diapausing *Ostrinia nubilalis* larvae induced supernumerary larval ecdysis, and the effect has been correlated with a high systemic JH titre [20]. In this paper we describe the effects of RH 5849 on the development of final instar *G. mellonella* larvae. We have found that RH 5849 has a typical juvenilizing effect in this insect species, eliciting the formation of perfect supernumerary larvae. We are particulary interested in the effects of RH 5849 on the activity of the brain corpora cardiaca/corpora allata axis during transition from the metamorphic to larval developmental programme.

## *Materials and methods*

Experiments were performed on the wax moth, *Galleria mellonella* (Lepidoptera, Pyralidae). The larvae were reared in constant darkness at 30 °C on a semi-artificial

diet[21]. The final (7th) instar larvae collected within 12 ± 6 h after ecdysis were designated as 1-day-old larvae. The non-steroidal ecdysone mimic, RH 5849 (1,2 dibenzoyl-1-t-butyl hydrazine) was a generous gift from Dr K. Wing, Rohm & Haas Company, USA. It was dissolved in 100% ethanol and doses of 0.34–150.0 µg in 1–5 µl of solvent were applied topically to the larvae. FMev (fluoromevalonolactone, gift from Sandoz, Zoecon Research Institute, Palo Alto, USA) was applied at a dose of 100 µg per larva in 1 µl of acetone. Nerve cord severance or bilateral removal of the corpora cardiaca/corpora allata complex was carried out by making a small incision in the cuticle on the ventral side of the neck. The nerve cord was cut between the suboesophageal and the first thoracic ganglia. The wound was then sealed with a drop of melting wax or rubber cement. Allatotropic activity of the brain was measured as described by Sehnal and Granger[8]. Brains were removed from water-anesthetized larvae into insect saline[22] and after 15–30 min implanted into the haemocel of 1-day-old final instar larvae. The allatotropic activity of two brains which induce 50% of supernumerary larval moults following implantation into host larvae has been defined as one allatotrophic activity unit (1 ATU). In in vitro studies the brain/corpora cardiaca/corpora allata complex was incubated for 3 h at 30 °C in Grace's medium containing 30 pg–30 ng of RH 5849 per 1 µl medium. Thereafter the complex was washed several times in insect saline, the corpora cardiaca/corpora allata cut off and the brain implanted into the haemocel of the larval host. The rate of JH synthesis was measured as described previously[23]. Four pairs of corpora cardiaca/corpora allata were incubated in 100 µl medium with 1 µCi of $^3$H-methyl-methionine (Amersham, final specific activity 9.8 Ci/mM). After 3 h incubation at 30 °C, the JH was quantified by means of the partition assay[24].

*Results and discussion*

RH 5849 is the first non-steroidal ecdysone agonist to mimic the action of this hormone by inducing premature cuticle synthesis[17–19]. It also competes with ponasterone A for high-affinity ecdysone receptor sites[16]. Premature apolysis of old larval cuticle and synthesis of a new one also occurred in penultimate and final instar larvae of *G. mellonella* treated with a single, high dose of RH 5849. Approximately 20% of 1-day-old penultimate instar larvae which received 1.75 µg of this compound slipped their head capsules within 18 h of RH 5849 application, and 1–2 days later most of them died without shedding their old cuticle. The response of the final instar larvae to RH 5849 was dependent on the age of larvae as well as on the dose applied. One-day-old final instar larvae which received 2.5–6.0 µg of RH 5849 moulted to supernumerary (8th) instar larvae at 3 ± 0.5 days after treatment: the maximal response (approximately 50% of supernumerary larval moults) was obtained with a dose of 6.0 µg per larva (fig. 1 A). The larvae which did not go

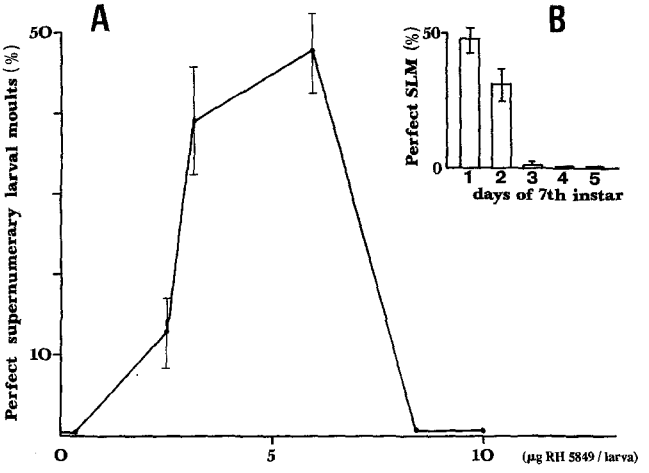


Figure 1. *A* Dose-response induction of perfect supernumerary larval moults of 1-day-old final instar larvae. *B* Age-dependent changes in sensitivity of the last instar larvae to RH 5849. Sensitivity is expressed in percent of supernumerary larval moults. Each larva received 6.0 µg of RH 5849 in 1 µl of ethanol. Bars represent the mean value of 3–7 replicates, each carried out on 15–26 larvae. Vertical lines indicate ± SD.

through supernumerary larval ecdysis after treatment with doses of 6.0 µg or less developed in a manner similar to controls and pupated after 7–8 days. Only a few of them (7%) formed larval-pupal intermediates. Doses equal to or greater than 8.5 µg of RH 5849 per larva caused premature formation of larval cuticle and were lethal. The sensitivity of the final instar larvae to RH 5849 declined sharply between day 2 and day 3 of the final instar since the dose of 6.0 µg of this ecdysone mimic per larva stimulated 31% of 2-day-old and only 2.1% of 3-day-old final instar larvae to supernumerary larval ecdysis (fig. 1 B). However, doses of 30 µg and 60 µg of RH 5849 applied on 3-day-old final instar larvae stimulated premature formation of larval cuticle (with or without small spots of the pupal cuticle) in 9.5% and 100% of individuals respectively. All of them died 1–2 days after new cuticle secretion (fig. 2 A, B). Precocious formation of pupal cuticle on the head, thorax and dorsal abdomen, and premature development of wings, imaginal legs, antennae and mouth apparatus occurred within 2 days following treatment of 4-day-old last instar larvae with 150 µg of RH 5849 (fig. 2 D). The dose of 60 µg per larva of this compound applied to 4-day-old final instar larvae caused formation of larval-pupal intermediates (34.4%). See figure 2 C.

Premature apolysis in all insect species examined so far, including *G. mellonella*, is associated with insect death a few days after treatment[15]. Evidently, high doses of the ecdysone mimic can induce precocious secretion of the cuticle but, like ecdysone, cannot speed up morphogenesis beyond certain limits without pathophysiological consequences[25]. The type of cuticle secreted by the epidermal cells of *G. mellonella* – larval type in young final instar larvae or larval-pupal composite type or pupal cuticle in 3–4-day-old final instar larvae – depends on the

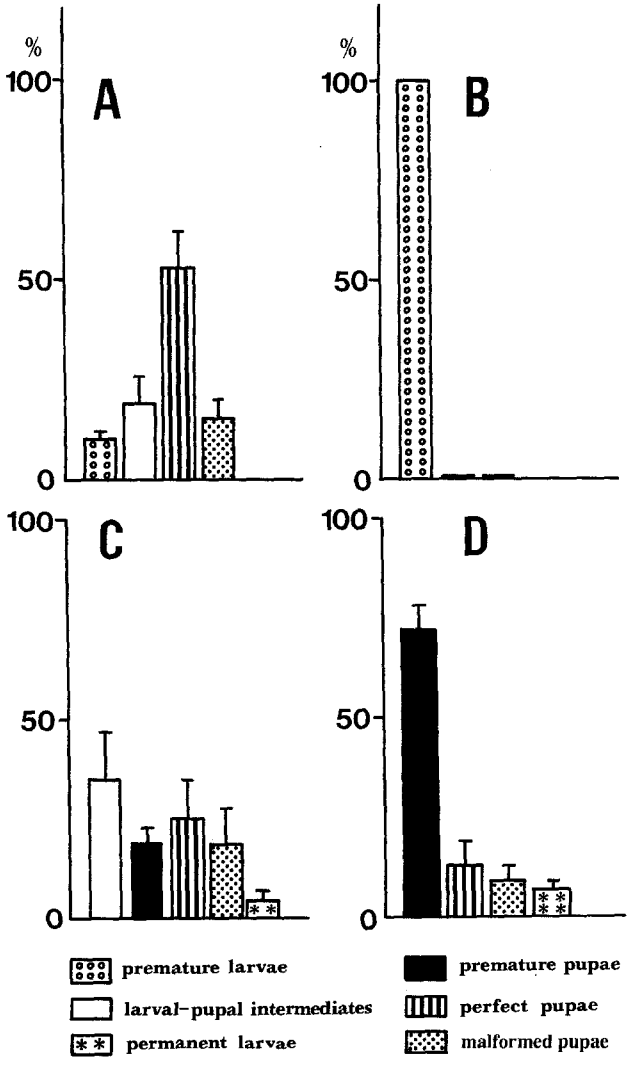


Figure 2. Morphological effect of high doses of RH 5849 on 3-day- and 4-day-old final instar larvae. Each 3-day-old larva received 30 µg (*A*) or 60 µg (*B*), and each 4-day-old larva received 60 µg (*C*) or 150 µg (*D*), of RH 5849. Premature formation of cuticle occurred within 1–2 days following RH 5849 application. Malformed pupae had a few spots of larval cuticle. Each bar represents the mean value of 2–3 replicates, each consisting of 10–15 larvae. Vertical lines indicate ± SD.

age of the larvae at the time of RH 5849 application. This supports the general rule that the secretory capacity of the epidermal cells is related to determination of pupal synthesis in the course of the final larval instar[26].

It has been shown in Lepidoptera that an increase in the JH titre during the sensitive period (generally the mid-point of the feeding period) of the final larval instar causes the larva to revert from the metamorphic to the larval development programme[27]. Information concerning the high JH titre is integrated with other internal and external stimuli which tune the brain and prothoracic glands for the larval programme of regulatory activity. In the brain this programme includes release of prothoracicotrophic hormone (PTTH) in the presence of JH[28]. Within the prothoracic gland, JH titre determines activation of the larval cycle of ecdysteroid release[29]. Application of moderate doses of RH 5849 to young final instar *O. nubilalis*[20] and *G. mellonella* larvae had a juvenilizing effect, since these insects underwent supernumerary larval ecdysis. The question is whether supernumerary larvae are the result of hyperecdysonism[30] or whether RH 5849 induces the juvenilizing effect in *G. mellonella* by its allatotropic activity. Our data support the second hypothesis. First, RH 5849-induced supernumerary larval ecdysis was dependent on de novo synthesis of JH, since bilateral allatectomy or treatment of larvae with 100 µg of FMev, markedly reduced the incidence of supernumerary larval ecdysis elicited by RH 5849 (table). Sec-

The effect of bilateral allatectomy and application of FMev on the supernumerary larval moults of 1-day-old final instar *G. mellonella* induced by RH 5849. The doses of RH 5849, FMev and hydroprene applied were 6.0 µg, 100 µg and 10 µg per larva, respectively.

| Treatment | No. animals tested/ fraction survived | % of supernumerary larval moults |
|---|---|---|
| Chilling | 52/50 | 67.1 |
| Chilling + FMev | 12/12 | 0.0 |
| RH 5849 | 36/32 | 58.3 |
| RH 5849 + FMev | 42/36 | 0.0 |
| Allatectomy | 15/12 | 0.0 |
| Allatectomy + RH 5849 | 21/16 | 12.5 |
| Allatectomy + hydroprene | 20/18 | 83.6 |

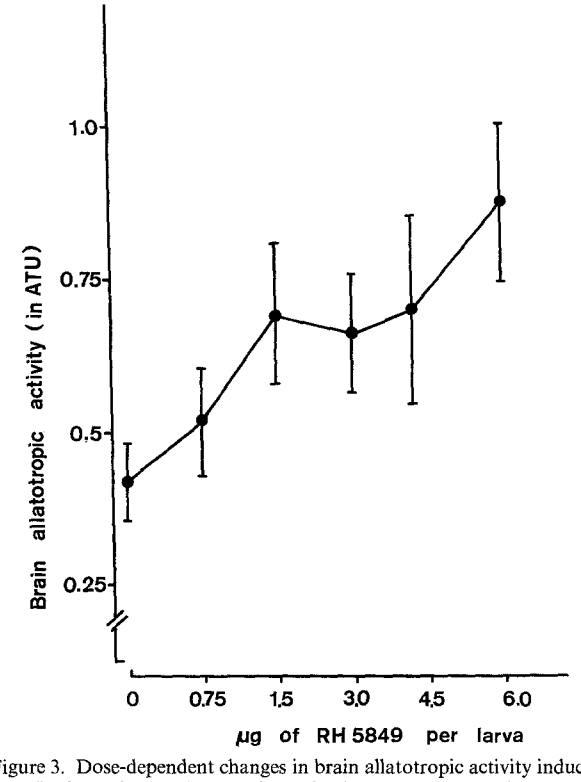


Figure 3. Dose-dependent changes in brain allatotropic activity induced by RH 5849. The activity was determined by an in vivo *Galleria* assay[8] 6 h after topical application of ecdysone mimic to 1-day-old final instar larvae. The brain activity which induced 50% of supernumerary larval moults following implantation into 1-day-old final instar larvae has been considered to represent one allatotropic activity unit (1 ATU). Each point represents the mean value of 2–4 independent measurements, each consisting of 12–15 larval hosts. Vertical lines indicate ± SD.

ond, RH 5849 caused an increase in brain allatotropic activity, i.e. the ability of the brain to activate JH synthesis by corpora allata. Dose-dependent induction of brain allatotropic activity by RH 5849 is shown in figure 3. This activity significantly increased following administration of a dose equal to or greater than 1.5 µg of RH 5849 per 1-day-old final instar larva; e.g. application of 3.0 µg and 6.0 µg of RH 5849 per larva caused an increase in the allatotropic activity of the brain to 0.66 ± 0.09 ATU and 0.88 ± 0.12 ATU, while the brain activity of untreated controls was 0.35 ± 0.06 ATU. Time-dependent studies of RH 5849-induced changes in brain allatotropic activity showed that 3 h after application of 6.0 µg of RH 5849 on 1-day-old final instar larvae, brain activity increased nearly 2.5 times, i.e. from 0.44 ± 0.08 to 1.1 ± 0.14 ATU. Thereafter allatotropic activity slowly decreased. However, even two days after RH 5849 application, activity was still significantly higher than that shown by untreated controls (fig. 4). Third, application of RH 5849 increased JH biosynthesis by corpora cardiaca/corpora allata. The in vitro rate of JH synthesis by corpora cardiaca/corpora allata obtained from larvae treated with RH 5849 (dose 6.0 µg per larva) was 89.1 ± 19.2 fmol/h/gland pair. This rate was nearly 2 times higher than that exhibited by the glands of ethanol treated controls (fig. 5). Taken together these data indicate that RH 5849 applied midway in the feeding period of the last larval instar, stimulates supernumerary larval ecdysis of *G. mellonella* by activating the brain corpora cardiaca/corpora allata axis.

It has been shown in vitro that the brain corpora cardiaca complex of *M. sexta* final instar larva is sensitive to

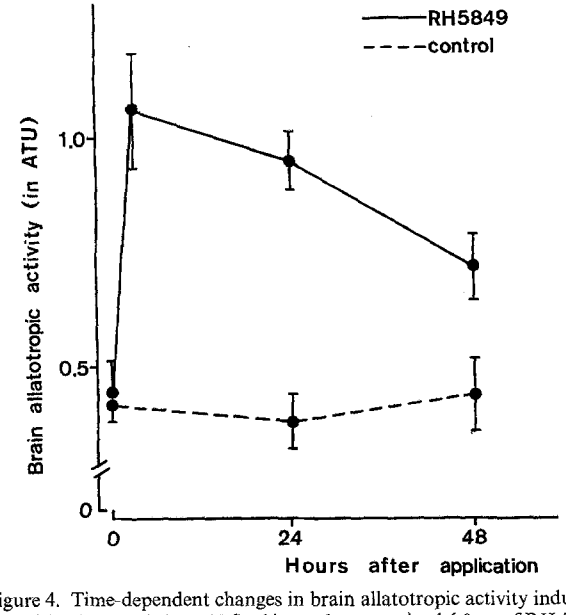


Figure 4. Time-dependent changes in brain allatotropic activity induced by RH 5849. Each 1-day-old final instar larva received 6.0 µg of RH 5849 in 1 µl of ethanol. Each point represents the mean value ± SD of 3 independent measurements, each consisting of 15–20 larval hosts. Other explanations as in figure 2.

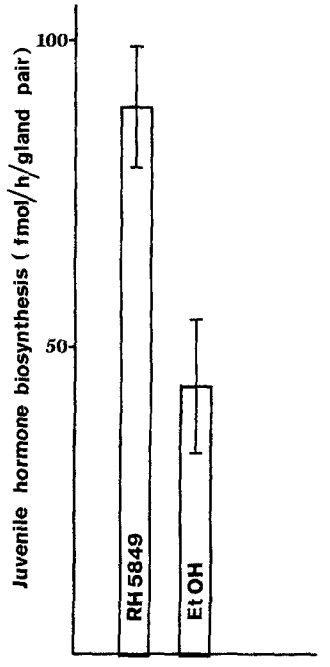


Figure 5. In vitro rate of juvenile hormone synthesis by corpora cardiaca/corpora allata complexes from 1-day-old final instar larvae treated with 6.0 µg of RH 5849 in 1 µl of ethanol. Control glands were obtained from larvae which received 1 µl of solvent. Each bar represents the mean value of 3–4 replicate determinations. Vertical lines indicate ± SD.

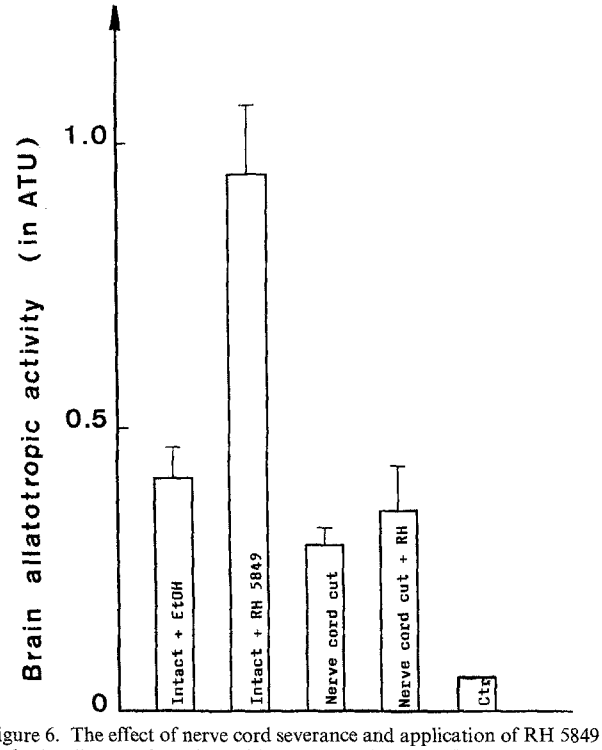


Figure 6. The effect of nerve cord severance and application of RH 5849 on brain allatotropic activity. The nerve cord was cut between the suboesophageal and the first thoracic ganglia. After operation, each 1-day-old final instar larva received 6 µg of RH 5849 in 1 µl of ethanol or 1 µl of solvent. Allatotropic activity of the brain is expressed in allatotropic activity units (ATU). The allatotropic activity of two abdominal ganglia (ct) obtained from RH 5849-treated larvae was below 0.1 ATU. Each bar represents the mean value of 2–3 replicate determinations.

20-hydroxyecdysone applied to the incubation medium at a strictly defined concentration of 20 ng per 1 ml medium, corresponding to the concentration of this hormone in the haemolymph of this instar larva[31]. At the above concentration 20-hydroxyecdysone activated JH synthesis by corpora allata indirectly via the brain corpora cardiaca axis[31]. The data obtained show that isolated *G. mellonella* larval brain is not sensitive to RH 5849. Severance of the nerve cord between the suboesophageal and the first thoracic ganglia abolished the stimulating effect of 6.0 µg RH 5849 per larva on the brain allatotropic activity (fig. 6). Furthermore, brain allatotropic activity did not increase when brain was incubated in vitro in Grace's medium in the presence of RH 5849 at a concentration of 30 pg–30 ng per 1 µl medium (not shown). The first ecdysteroid peak, probably responsible for activation of brain allatotropic activity and induction of the supernumerary larval moult of *G. mellonella*, reached a value of 30 pg per 1 µl of haemolymph[32]. It has been shown that chilled larvae did not undergo supernumerary ecdysis when their nerve cord was cut between the suboesophageal and the first thoracic ganglion[7], which strongly suggests that information on the ambient temperature is transmitted from the peripheral thermoreceptors to the brain via the ventral nerve cord[33]. In beehives, the natural habitat for the wax moth, changes in ambient temperature are relatively low, and exposure to a temperature of 0 °C is a powerful stress factor for this insect species[33]. It seems that RH 5849-induced supernumerary larval moult of the young final instar larvae of *G. mellonella* can also be considered as a stress-induced effect since it occurred when moderately high doses of this ecdysone mimic were applied, i.e. 2.5–6 µg per larva. Thus, the titre of RH 5849 in haemolymph of the treated larvae is probably much higher than the concentration of ecdysteroid in haemolymph of young *G. mellonella* larvae[2,6].

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