Eur. J. Entomol. **92**: 325–332, 1995
ISSN 1210–5759

Age-specific effects of a non-steroidal ecdysteroid agonist, RH-5992, on the spruce budworm, *Choristoneura fumiferana* (Lepidoptera: Tortricidae)

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***Choristoneura*, RH-5992, molting, CHR3, ecdysteroids, 20-hydroxyecdysone, MHR3**

Abstract. The non-steroidal ecdysone agonist, RH-5992, is effective in inducing an incomplete molt in the spruce budworm, *Choristoneura fumiferana* (Clemens), only when it is fed to the larvae prior to the appearance of the endogenous ecdysteroid peak. When this compound is administered after the ecdysteroid peak, the larvae molt normally into the next stage. However, because of its persistence, the agonist induces an incomplete molt in the subsequent larval stage. The effect does not appear to be due to ecdysone responsive tissues becoming refractory after the ecdysteroid peak. *Choristoneura* hormone receptor 3 (CHR3), a homologue of *Manduca* hormone receptor 3 (MHR3), is induced in the epidermis, fat body and midgut of 6th instar larvae treated with RH-5992 during all days of the 6th stadium. Possible reasons for this age specific effect are discussed.

INTRODUCTION

The non-steroidal ecdysteroid agonist, RH-5992, has been shown to act similar to ecdysteroids in initiating the molting process in lepidopterans such as *Manduca sexta* (Retnakaran et al., 1994) and *Choristoneura fumiferana* (Retnakaran & Oberlander, 1993). Similar to 20-hydroxyecdysone (20E), RH-5992 induces the *Manduca* hormone receptor 3 (MHR3) mRNA, suppresses 14 kDa larval cuticular protein (LCP-14) mRNA and prevents the expression of dopadecarboxylase (DDC) mRNA because of its persistence (Retnakaran et al., 1994).

Upon ingestion of RH-5992, within 24 hr the insect retracts its muscles from the integument, becomes quiescent and stops feeding. The larva goes through an incomplete molt and dies in this state (Binnington & Retnakaran, 1991). The present study is designed to determine whether or not the effects of RH-5992 are stage specific and influenced by endogenous levels of ecdysteroids during larval life.

In this paper we show that RH-5992 is effective in inducing a precocious incomplete molt resulting in mortality, only if the spruce budworm larva is treated during the first 4 days of the 6th stadium. Application of this compound after the ecdysteroid peak on day 4 of the 6th instar, does not interfere with the normal molt. The same is true in the 5th instar larvae as well where the effect is evident when treated on the first 3 days but not on the subsequent 2 days. However, because of its persistence, the compound causes its molt-inducing effects on the 6th instar larva.

Manduca hormone receptor 3 is a member of the steroid hormone receptor superfamily and MHR3 mRNA appears in the *Manduca* epidermis only during ecdysteroid rises for embryonic, larval and pupal molts. The MHR3 gene is known to be induced by

20-hydroxyecdysone (20E) (Palli et al., 1992). RH-5992 also induced MHR3 both in vivo and in vitro in the *Manduca* epidermis (Retnakaran et al., 1994). We have recently cloned the MHR3 homologue from the spruce budworm and referred to it as *Choristoneura* hormone receptor 3 (CHR3) (Palli et al., in prep.). Using the induction of CHR3 gene as an indicator of ecdysone responsiveness of cells, we show that the midgut, fat body and epidermal cells are responsive to RH-5992 from the beginning to the end of *Choristoneura fumiferana* larval stadium indicating that it is not the refractiveness of the cells to RH-5992 but interference with the normal sequence of events during the molting process that is responsible for the observed molt induction of this compound.

MATERIAL AND METHODS

Experimental animals

Spruce budworm larvae [*Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae)] were reared on artificial diet (McMorran, 1965) at 22°C, 70% relative humidity and a photoperiod of 12 : 12 hr light and darkness as described in Grisdale (1970). Larvae were selected as soon as they molted into either the 5th instar or 6th instar larvae, when the head was still untanned (white head) and remained immaculate for about 30 min.

Ecdysteroid assay

Staged 6th instar larvae were immobilized on ice and one microliter of hemolymph per larva was collected and extracted twice with methanol, evaporated to dryness and taken up in the phosphate-EDTA buffer provided with the 20-hydroxyecdysone (20E) competitive enzyme immunoassay kit (Cayman Chemicals, Ann Arbor, MI, USA). The assay was performed as described in the protocol provided by Cayman Chemicals (Maxey et al., 1992).

In vivo assay for RH-5992 effects

Technical RH-5992 (99% pure) was dissolved in isopropanol at 20 ng/µl and one microliter was deposited on a 5X1 mm cylinder diet plug and fed to individual larvae in a microtiter plate during a period of ≤ 2 hr. Control larvae received one microliter of isopropanol. Both treated and control larvae were maintained at rearing conditions described earlier. The effects were observed daily after the treatment for 10 days.

CHR3 induction assay

Six hours after the larvae were treated with RH-5992, epidermis, fat body and midgut were dissected and the total RNA was isolated by guanidinium isothiocyanate-phenol-chloroform extraction method (Chomczyski & Sacchi, 1987). RNA concentrations were determined spectrophotometrically. Ten micrograms of denatured total RNA was spotted onto Hybond N membrane (Kafatos et al., 1979). The dot blots were probed with [^{32}P] labeled CHR3 cDNA probe. The hybridization and washing conditions are as described in Palli et al. (1992). The intensity of dots was analyzed by using the CAMAG evaluation program (CAMAG, Switzerland). CHR3 expression during embryonic molt was used as the standard. Ten micrograms of total RNA isolated during embryonic molt was spotted on to each blot and all the samples were compared to the standard which was set at 100%.

RESULTS

Ecdysteroid levels during 6th instar

As shown in Figure 1, the ecdysteroid levels remained low in the hemolymph for the first three days. A small peak is seen on day 4, which is followed by a larger peak on days 6–8. The pattern of ecdysteroid levels is similar to that reported in *Manduca sexta* (Bollenbacher et al., 1981; Curtis et al., 1984; Kato & Riddiford, 1987).

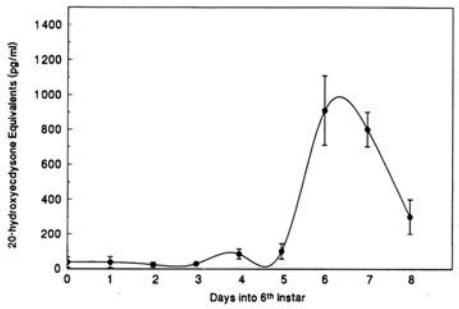

Fig. 1. Ecdysteroid levels during 6th instar spruce budworm, *Choristoneura fumiferana*. 20-hydroxyecdysone equivalents were measured in the hemolymph collected from staged 6th instar larvae using the competitive immunoassay kit from Cayman Chemicals (Ann Arbor, MI, USA). Each point represents an average of 6–8 larvae and the bars indicate S.E.

Stage specific effects of RH-5992

The typical phenotypic effects induced by RH-5992 are shown in Figure 2. Immediately after ecdysis the larva shows an untanned white head (Fig. 2A), which lasts for 30 min after which it turns dark brown (Fig. 2B). The RH-5992 fed larvae start showing effects 12 hr after treatment when they become quiescent and stop feeding (Fig. 2C). Within 24 hr

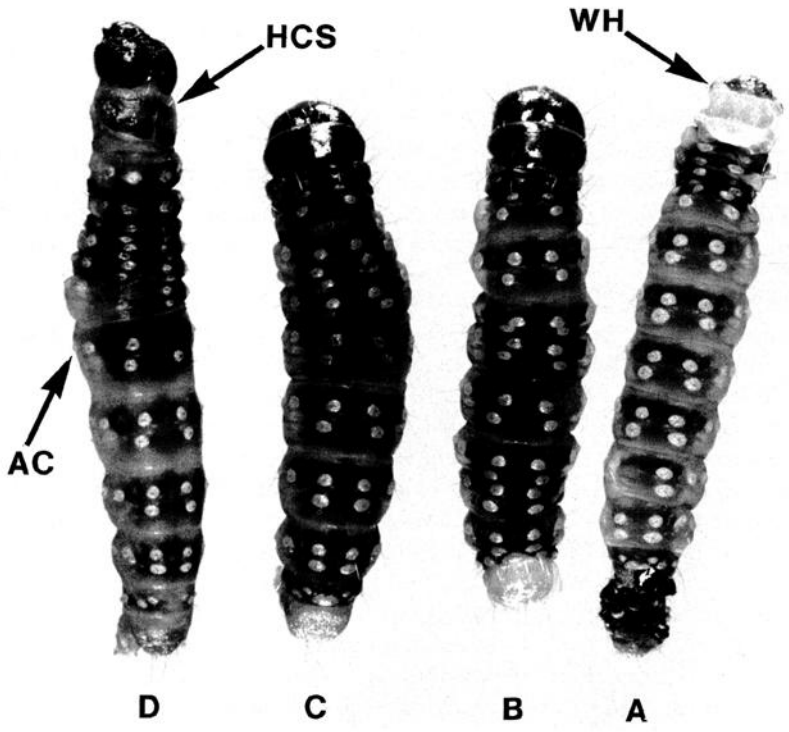

Fig. 2. Effect of RH-5992 on 6th instar spruce budworm, *Choristoneura fumiferana*. A – newly molted 6th instar larvae showing an untanned white head capsule (WH); B – sixth instar larva 30 min after molting showing a tanned dark brown head capsule at which time the larva was allowed to feed on a pellet of diet (5 mm long × 2 mm diameter cylinder) containing 20 ng of RH-5992; C – RH-5992 effect 12 hr after treatment showing very little phenotypic changes however, the larva stops feeding and becomes quiescent; D – RH-5992 effect 24 hr after treatment showing head capsule slippage (HCS) and a loose apolysed cuticle (AC). The larva remains at this stage and does not develop further or undergo ecdysis.

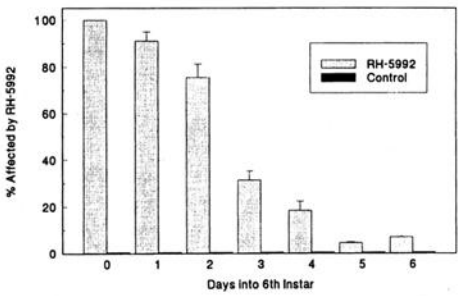

Fig. 3. Effect of RH-5992 on 6th instar spruce budworm, *Choristoneura fumiferana*. Twenty ng of RH-5992 in isopropanol was fed to 6th instar larvae on days 0–6 and the larvae were observed for head capsule slippage 24 hr later. The control larvae received isopropanol. Each treatment (n = 16) was replicated 4 times and the bars indicate S.E.

after treatment, the head capsule starts slipping for the molt and the apolysed cuticle appears loose (Fig. 2D). Unlike head capsule slippage in normal larvae which is followed by molting, treated larvae remain arrested at this stage without further development and ecdysis.

Sixth instar larvae from days 0–6 treated with 20 ng of RH-5992 showed varying degrees of effects (Fig. 3). One hundred percent of the larvae treated on day 0 showed head capsule slippage in 24 hr. The effect was slightly less (> 80%) on days 1 and 2. By days 3 and 4 there was significant reduction in the effect of RH-5992, only 20–30% of the treated animals showed head capsule slippage. After day 4 the effect was reduced to minimal levels (< 10%) and they pupated into normal pupae that did not show any effects. On the other hand, when 5th instar larvae were similarly treated on the first 3 days of the stadium, 100% of the larvae showed head capsule slippage within 24 hr whereas those that were treated on days 4 and 5 showed < 10% effect. But these larvae (i.e., treated on days 4 and 5), after the appearance of the ecdysteroid peak, molted normally into 6th instars but within 24 hr of molting, 100% of the larvae showed the characteristic incomplete molt.

CHR3 induction during 6th instar

In the epidermis, fat body and midgut isolated from RH-5992 treated larvae, CHR3 mRNA is induced in an hour and reaches maximum levels in 3–6 hr followed by a decline in 12–24 hr (results not shown). Since RH-5992 is effective only during the first 4 days of the 6th stadium, we wanted to determine whether or not the cells are refractive to this compound during the later part of the stadium when it is not effective. Epidermis, fat body and midgut were dissected from larvae 6 hr after ingestion of RH-5992, the total RNA was isolated and CHR3 mRNA levels were measured during the initial 7 days of the 6th stadium. Figure 4 shows that CHR3 mRNA levels increased after exposure to RH-5992 when compared to controls in all three tissues. In the epidermis (Fig. 4A) the CHR3 mRNA levels were significantly higher in RH-5992 fed larvae when compared to isopropanol fed larvae until day 6. On the final day of the 6th stadium, prior to pupation, CHR3 is induced in the epidermis in response to the prepupal ecdysteroid peak (Palli et al., in prep.). We can therefore conclude that there is really no significant difference in CHR3 mRNA levels between the treated and untreated larvae, the increased expression in day 6 controls being due to the back ground prepupal ecdysteroid peak. In the fat body (Fig. 4B) RH-5992 treated larvae showed significantly higher levels of CHR3 mRNA than controls during the entire 6th stadium. In the midgut (Fig. 4C), RH-5992 treated larvae showed significantly higher levels of CHR3 mRNA when compared to controls on days 0, 1, 2, 3 and 5. During

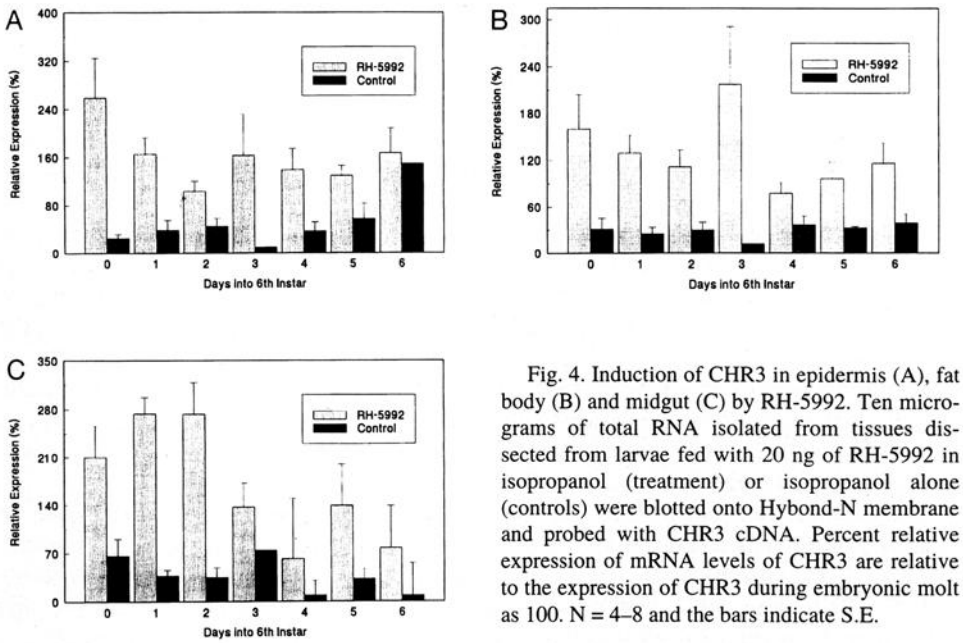

Fig. 4. Induction of CHR3 in epidermis (A), fat body (B) and midgut (C) by RH-5992. Ten micrograms of total RNA isolated from tissues dissected from larvae fed with 20 ng of RH-5992 in isopropanol (treatment) or isopropanol alone (controls) were blotted onto Hybond-N membrane and probed with CHR3 cDNA. Percent relative expression of mRNA levels of CHR3 are relative to the expression of CHR3 during embryonic molt as 100. N = 4–8 and the bars indicate S.E.

days 4 and 6 the levels were less than what was observed on the other days. The CHR3 mRNA is induced by RH-5992 in all three tissues on all days of the 6th stadium. The induction levels are not significantly different from each other on various days in the fat body and the epidermis but, in the midgut the induction levels during the final three days are lower when compared to those during the first three days.

DISCUSSION

The most significant finding reported in this paper is that RH-5992 induces an incomplete molt in the larvae only when it is ingested by the larvae during the early part of the stadium and is ineffective during the latter part of the stadium. Since this compound persists in the larva, treatment during the latter part of the 5th stadium manifests its effects on the first day of the subsequent 6th instar. Similar treatment of 6th instars has no visible effect on the subsequent pupal stage which appears to be refractory to this compound. In this paper we have examined some of the possible reasons for the age dependent effects and we focussed our efforts on the 6th instar stage.

The ecdysteroid levels during the last larval instar of the spruce budworm follow a pattern similar to that reported for other lepidopterans such as *Manduca sexta* (Bollenbacher et al., 1981; Curtis et al., 1984; Kato & Riddiford, 1987). In the spruce budworm we have detected a small ecdysteroid peak similar to the commitment peak on day 4 and a larger prepupal peak on days 6 and 7 reported in *Manduca sexta*. RH-5992 is highly effective during the first three days, then progressively becomes less effective in the subsequent two days after which it is almost ineffective. It appears, therefore, that RH-5992 is effective

only before the commitment peak of ecdysteroids whereas it is less effective once the tissues have been exposed to the ecdysteroids.

One possible reason for the effects observed could be due to the different tissues becoming refractory to RH-5992 once they are exposed to the endogenous ecdysteroids. To test this hypothesis, we have used CHR3, a homologue of MHR3, an ecdysone-induced DNA-binding protein as a marker. MHR3 mRNA is induced by 20E (Palli et al., 1992) as well as by RH-5992 (Retnakaran et al., 1994). We have used three different tissues, fat body, midgut and epidermis to study the induction of CHR3 by RH-5992 during the different days of the 6th larval stadium. CHR3 mRNA is induced in all three tissues during all the days although the midgut appears to be less sensitive after day 3. These data suggest that the cells remain competent and respond to RH-5992 in inducing CHR3 throughout the 6th instar.

Another reason for this age dependent effect of RH-5992 is the changes in the metabolic and detoxifying ability of the tissues to clear the compound as a function of age. However, this seems unlikely since the effect is observed in the subsequent stage when the compound is applied to the larva during the latter part of the previous stadium.

Ecdysteroids regulate molting and metamorphosis by controlling the expression of genes by different mechanisms. Some genes are not expressed in the presence of ecdysteroids (LCP-14, Hiruma et al., 1991), some gene expression is induced by ecdysteroids (MHR3, Palli et al., 1992) and still other genes require transient exposure for expression later after the hormone is cleared (DDC, Hiruma & Riddiford, 1990). When RH-5992 is fed to the larvae during early days, prior to the appearance of ecdysteroid peak, all the negatively regulated genes such as LCP-14 are not expressed. Genes that are induced by ecdysteroids are probably expressed and molting is initiated. Because RH-5992 persists in the system, genes such as DDC that require transient exposure will not be expressed. The result is the initiation of a molt which is not completed due to incomplete gene expression in the molting cascade. When RH-5992 is fed to the larvae after the tissues are exposed to ecdysteroids (after day 5), the larvae appear to develop normally and undergo a regular molt. In this instance all the genes negatively regulated by ecdysteroids are probably already expressed during the first four days. The positively regulated genes and the genes that require transient expression are also expressed since normal molting and tanning do occur. The normal ecdysis and related phenomena that occur in the larvae treated with RH-5992 late in the stadium remains enigmatic. The eclosion hormone stored in the proctodeal nerves is released only when the endogenous ecdysteroids are cleared from the system (Truman, 1992). Since RH-5992 persists in the larvae, it should prevent ecdysis. Contrary to this expectation, larvae treated during days 4–6 went through normal ecdysis similar to the control larvae. Different isoforms of ecdysone receptors present before and after the commitment peak of ecdysteroids as observed in *Drosophila* (Talbot et al., 1993) might have different binding affinities to RH-5992. Preliminary work in our laboratory has indicated that only larval stages of the spruce budworm are susceptible to RH-5992, pupal and adult stages showing no effects. Another possibility is that RH-5992 is unable to reinitiate a cascade of events once the natural ecdysteroid peak has initiated the molting sequence. These and other possible explanations are currently being examined to elucidate the possible reasons for the failure of RH-5992 to stop ecdysis when applied during the latter part of the larval stadium.

SUMMARY AND CONCLUSIONS

1. The non-steroidal ecdysteroid agonist, RH-5992, induces precocious incomplete molt in larvae that have ingested this compound during the early part of the stadium, before the appearance of the ecdysteroid peak.
2. Larvae treated with RH-5992 during the latter part of the stadium after the appearance of the peak, show no adverse effects and go through the normal molt.
3. CHR3 mRNA is induced by RH-5992 in epidermis, fat body and midgut throughout the 6th larval stadium indicating that these ecdysone responsive tissues remain competent throughout the stadium.
4. Changes in the metabolism or detoxification of RH-5992 after the appearance of the ecdysteroid peak may not explain the differences in the effect of RH-5992 during the stadium because of the carryover effect on the subsequent larval stage.
5. Events such as the release of eclosion hormone that require the decline of ecdysteroid titer are apparently unaffected by RH-5592 treatment during the latter part of the stadium. Appearance of different isoforms of ecdysteroid receptor that does not bind to RH-5992 or inability of RH-5992 to reinitiate a cascade of events leading to the release of the eclosion hormone at such a late stage in the stadium are some of the possible explanations.

ACKNOWLEDGEMENTS. We thank Dr W.M. Cheliak for discussions throughout the study and for critical reading of the manuscript. Supported by the Canadian Forest Service and National Biotechnology Strategy Fund.

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