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Interactions of Ecdysteroid and Juvenoid Agonists in *Plodia interpunctella* (Hübner)

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The influence of non-steroidal ecdysteroid agonists on Indianmeal moth larvae was assessed by rearing last instar larvae on diet treated with RH-5992 (tebufenozide) or RH-2485 (methoxyfenozide). Larvae were monitored for effects of the ecdysteroid agonists on weight, metamorphosis and mortality. Larvae treated with either of the ecdysteroid agonists at a concentration of 5 ppm or higher gained less weight and had greater mortality than did larvae reared on control diet. For example, the weights of control larvae increased approximately 400% by day 2, compared with only a 50% increase in weight when the larvae were treated with 25 ppm of RH-2485 or RH-5992. Similarly, mortality in control larvae was less than 10%, but was as much as 90–100% in larvae reared on diet treated with one of the ecdysteroid agonists. We also examined the effects of simultaneous treatment with a juvenile hormone (JH) mimic, either methoprene or fenoxycarb. The JH mimics prevented adult emergence, and the larvae continued to feed throughout the month-long observation period. However, larvae treated with a juvenile hormone mimic gained weight despite the presence of an ecdysteroid agonist in the diet. On diets treated with 0.1 ppm of RH-2485 or RH-5992, JH-treated larvae gained even more weight than did untreated controls. Interestingly, although the addition of a JH mimic to ecdysteroid-treated diet resulted in increased weight, it did not lead to reduced mortality. In fact, combinations of a JH mimic with 10 ppm RH 2485 or RH 5992 resulted in nearly 100% mortality compared with 40–70% mortality without the JH compounds. These results indicate that JH mimics overcome the inhibitory effects of ecdysteroid agonists on weight gain; however, they also resulted in increased mortality compared with moderate doses of ecdysteroid agonists alone. One specific action of these compounds at the cellular level was noted in that RH 5992 mimicked ecdysteroids by increasing uptake of ^{14}C-GlcNAc in a *Plodia interpunctella* cell line, while fenoxycarb was inhibitory. Arch. Insect Biochem. Physiol. 38:91–99, 1998. © 1998 Wiley-Liss, Inc.†

Key words: dibenzoyl hydrazine; fenoxycarb; methoprene; RH-5992; RH-2485; tebufenozide; methoxyfenozide

Abbreviations: F = fenoxycarb; GlcNAc = N-acetyl glucosamine; JH = juvenile hormone; M = methoprene; ppm = part per million.

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INTRODUCTION

During the past decade, a new class of insect growth regulators has become available with the discovery that a series of dibenzoyl hydrazine compounds act as agonists of 20-hydroxyecdysone (Wing, 1988, Wing et al., 1988). Attention was first focused on laboratory studies of RH-5849, as well as on the more potent compound, RH-5992 (tebufenozide), which shows promise against Lepidoptera in agricultural applications (Oberlander et al., 1995). Recently, another active compound in this series, RH-2485, methoxyfenozide, (N´ (3-methoxy-2-methylbenzoyl)-N-(3.5-dimethylbenzoyl)-N-t-butylhydrazine), has been made available for investigational purposes (Trisyono and Chippendale, 1997).

As a result of the development of resistance to insecticides by stored product insects and the limitations on the use of fumigants due to regulatory and environmental considerations, there is a continuing interest in the evaluation of insect growth regulators as protectants for stored commodities. Juvenile hormone (JH) agonists have been investigated most thoroughly in this regard and have displayed effectiveness against many stored product Coleoptera and Lepidoptera (see reviews by Bengston, 1987; Bengston and Strange, 1994; Oberlander et al., 1997). These hormone mimics combine the advantages of low mammalian toxicity, target specificity, and compatibility with other chemical and biological treatments. In addition to JH agonists, chitin synthesis inhibitors are active against a variety of stored product insects including, *Plodia interpunctella* (Hübner), *Sitophilus oryzae* (L.) *Sitophilus granarius* (L.), and *Tribolium castaneum* (e.g., McGregor and Kramer, 1975; Desmarchelier and Allen, 1992; Elek, 1994; Elek and Longstaff, 1994; Oberlander et al., 1997).

Despite the substantial work with agricultural applications of ecdysteroid agonists to field crop pests, there has been little attention paid to the actions of these types of compounds on stored product insects. Research in our laboratory on various effects of RH-5849 on *P. interpunctella* in vitro and in vivo, demonstrated the range of similarities between the actions of 20-hydroxyecdysone and this ecdysteroid agonist (Silhacek et al., 1990). Moreover, as part of these studies we demonstrated that simultaneous treatment of RH-5849 and the JH mimic, methoprene, prevented the reduction in weight gain that would otherwise have been caused by RH-5849 (Silhacek et al., 1990). This result was confirmed in *Spodoptera exigua*- (Hübner) (Smagghe and Degheele, 1994).

In the present work we have expanded our investigation of the actions of ecdysteroid agonists to include RH-5992 (tebufenozide) and RH-2485 (methoxyfenozide), and we have determined their effects on larval growth and mortality of *P. interpunctella.* In addition, we have investigated the impact of simultaneous treatment of larvae with the JH mimics, methoprene and fenoxycarb, and these ecdysteroid agonists to establish more fully the nature of their interactions during the extended larva stage induced by the JH compounds. Finally, we utilized a *P. interpunctella* cell line to test the effects of fenoxycarb and RH 5992 on GlcNAc uptake, a process that is linked to chitin synthesis.

MATERIALS AND METHODS

P. interpunctella was reared on a cereal diet according to Silhacek and Miller (1972). Larvae immediately after the last larval molt were reared continuously on diet that was treated with RH-5992 or RH-2485 (courtesy of Rohm and Haas Co.) as well as methoprene (Altosid, 65.5% AI, Zoecon Corp., NOVARTIS, Basle, Switzerland) or fenoxycarb (Maag Agrochemicals, NOVARTIS, Basle, Switzerland). Test quantities of the compounds were dissolved in 5 ml of acetone and mixed with a 7 g portion of the dry diet components. The acetone was evaporated from the diet, and the dried test diet was mixed with 3 g of honey-glycerol. Finally, 10 g of the complete treated diet was divided among five 25 cm^2 plastic boxes, and 10 newly molted final instar larvae were added to each box. The larvae were held in an incubator at a 16:8 photoperiod at 30°C and 70% R.H. The test larvae were examined daily for weight gain and mortality until metamorphosis and then weekly for a maximum of 31 days in the case of larvae treated with juvenile hormone agonists.

The data were analyzed by analysis of variance and regression analysis (SAS; Littell et al, 1991). The growth data were analyzed with respect to weight as a function of duration of treatment (day) and concentration of both the ecdysteroid and JH agonists. The data for the impact of specific ecdysteroid and JH agonists at various concentration and on different days of observation were described by non-linear (quadratic) regression. For example, in one experiment the 5-term equation for weight of larvae was: Weight = 3.64 – 0.5[RH-5992 concentration] + 6.13[Day] –0.19[RH-5992 concentration × Day] + 0.02[RH-5992 concentration]^2 –0.95[Day]^2; while cumu-

lative mortality was described by the equation: Mortality = 5.64 + 8.94[RH-5992 concentration] – 0.21[RH-5992 concentration]^2. For statistical analysis of our experiments interactions of day of treatment, concentration of ecdysteroid, and concentration of juvenoid were included in the statistical model and tested for significance. The nonadjusted R^2 and P values that describe the data are presented in the text. In general, the results of the experiments depended upon the concentration of the agonist and the duration of treatment. The data are displayed in the form of histograms. Mortality of larvae was recorded for insects treated with ecdysteroid agonists only during the 3-day period prior to metamorphosis, or during the course of 31 days of observation in the case of larvae treated with both ecdysteroid and JH agonists.

For the in vitro tests IAL-PID2 cells were maintained in modified Grace's medium. Uptake of ^{14}C-GlcNAc (2.0 μCi/flask) by the cells was measured as previously described (Porcheron et al., 1988; Silhacek et al. 1990).

RESULTS

Effects of RH-5992 and RH-2485 on Growth

In the first experiment newly molted last instar larvae were transferred to diet treated with various concentrations of ecdysteroid agonist. Untreated larvae gained weight during the first 2 days, and then their weight gain leveled off prior to the onset of metamorphosis on day 4. Larvae reared on diet with concentrations of RH-5992 of less than 1.0 ppm gained weight in a manner similar to the untreated larvae. However, at concentrations of 5.0 ppm or higher of RH-5992 the weight gain typical of larval development was not observed, while the highest concentration of RH-5992 tested led to reduced larval weight (Fig. 1A). Thus, the weight of untreated larvae increased approximately 500% by day 3, compared with a slight decrease in weight when the larvae were reared on diet that was treated with 25 ppm of RH-5992. Statistical analysis showed that the weight of the larvae depended with a high degree of significance on both the day of treatment ($P < 0.0001$) and on the concentration of RH-5992 ($P < 0.001$). When the data for all days and concentrations were combined and analyzed, there was a joint effect of concentration of RH-5992 and day of treatment on weight ($R^2 = 0.87$, $P < 0.0001$).

In the second experiment, last instar larvae were reared on diet treated with RH-2485, and their weights were monitored daily. The weight of control larvae increased approximately 400% over 3 days, while those larvae treated with 10–25 ppm RH-2485 increased by only approximately 50%. However, larvae exposed to 0.1 ppm RH-2485 weighed more than untreated larvae on days 2 and 3 (Fig. 2A). Thus, as with RH-5992, the weight of the larvae depended upon the concentration of RH-2485 in the diet. However, RH-2485 was less effective than RH-5992 in preventing weight gain of the treated larvae. Statistical analysis supported the conclusion that the weight of the larvae depended upon the concentration of RH-2485 in the diet ($P < 0.0001$). Moreover, when the data for all days and concentrations were considered together there was a joint effect of concentration and day on weight that was highly statistically significant ($R^2 = 0.77$, $P < 0.0001$).

Larvae reared on diet treated with various concentrations of RH-5992 and 10 ppm methoprene did not metamorphose and were monitored for 31 days. Note that the data from representative days of observation (Figs. 1 and 2) do not show weights for larvae at the highest concentrations of ecdysteroid agonists at later observation periods because of mortality of the larvae, as described in Effects of RH 5992 and RH 2485 on Mortality. The larvae continued to gain weight during the period of observation at concentrations of RH-5992 up to 10 ppm. However, at higher concentrations of RH-5992, in the presence of methoprene, there was a diminished weight gain over time, though the treated larvae still weighed more than untreated controls (Fig. 1B). Similar results were obtained when RH-2485 and methoprene were applied together (Fig. 2B) . These results are described by a quadratic regression ($R^2 = 0.94$ for both the RH-5992 and RH-2485 data, $P < 0.0001$ for each).

The effects of the ecdysteroid agonists on weight of treated larvae also were measured in the presence of fenoxycarb. As with methoprene, during the month-long observation period fenoxycarb prevented metamorphosis of the larvae. When last instar larvae were reared on diet treated with either RH-5992 or RH-2485 and 5 ppm fenoxycarb, there was a 4-fold increase in weight compared with untreated larvae just prior to pupation; e.g., compare day 3 larvae (0 ppm, Fig. 1A and Fig. 2A) with day 22 larvae treated with 1 ppm of ecdysteroid agonist and 5 ppm fenoxycarb (Fig. 1C and 2C). However, as with the methoprene experiment, this additional weight gain was not as high when the concentration of ecdysteroid agonist exceeded 5 ppm. Sta-

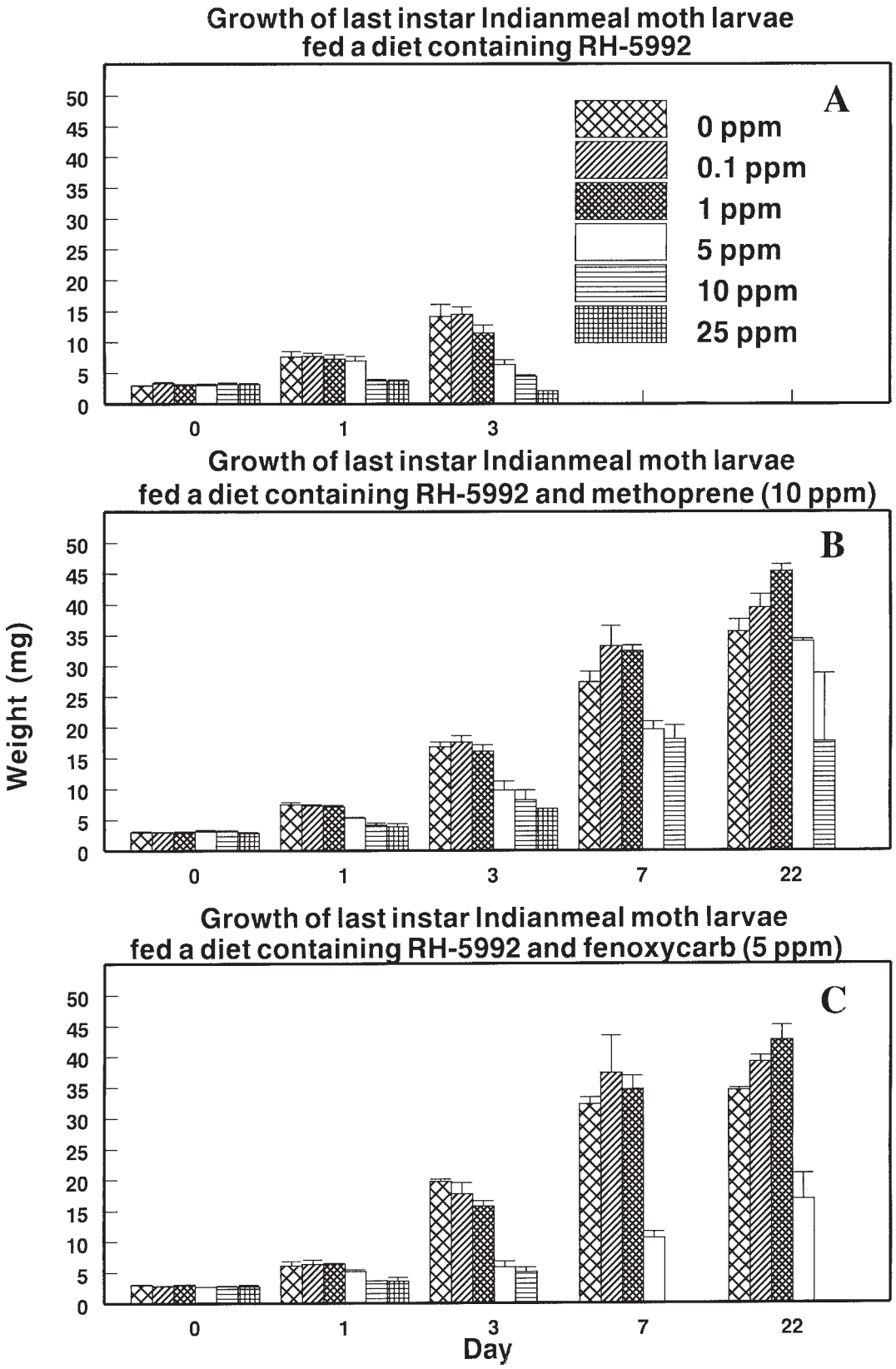

Fig. 1. Weight in mg (± S.E.) of last instar Indianmeal moth larvae fed a diet of RH-5992 alone or in combination with either fenoxycarb or methoprene at the concentrations indicated. **A:** RH-5992. **B:** RH-5992 and methoprene. **C:** RH-5992 and fenoxycarb. Mortality of larvae at the higher concentrations and longer duration of treatment resulted in the absence of data at these combinations.

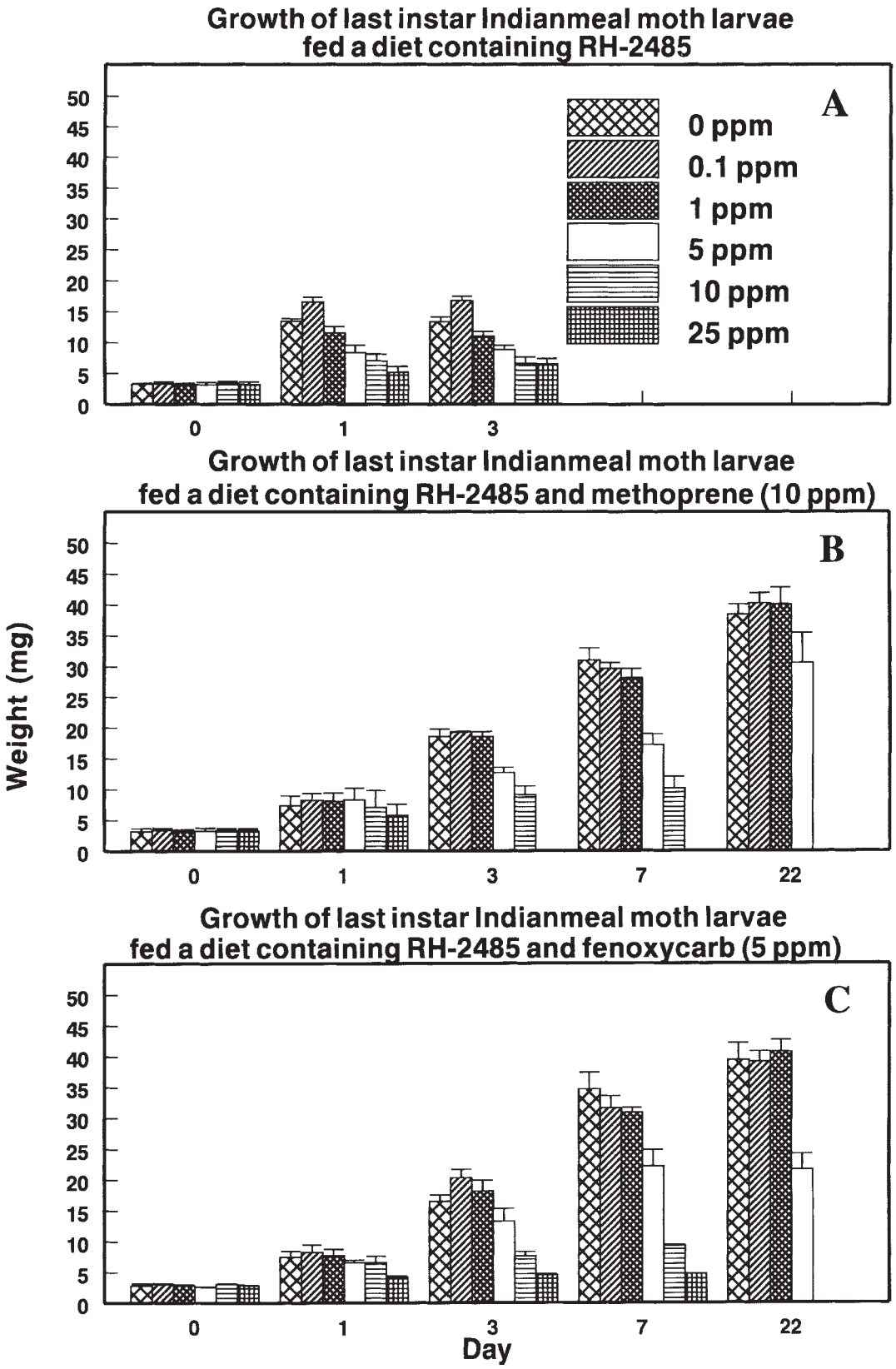

Fig. 2. Weight in mg (± S.E.) of last instar Indianmeal moth larvae fed a diet of RH-2485 alone or in combination with either fenoxycarb or methoprene at the concentrations indicated. **A:** RH-2485. **B:** RH-2485 and methoprene. **C:** RH-2485 and fenoxycarb. Mortality of larvae at the higher concentrations and longer duration of treatment resulted in the absence of data at these combinations.

tistical analysis showed that the dependence of larval weight upon the concentration of ecdysteroid agonist and the number of days of treatment was highly significant ($P < 0.0001$ for each). The dependence of larval weight on treatment with different concentrations of RH-5992 or RH-2585 and fenoxycarb was highly significant (R^2 of 0.89, R^2 of 0.95, respectively; $P < 0.0001$ for each). Finally, statistical analysis showed that when the combined data for all concentrations of ecdysteroid agonist in the presence of JH agonist, and days of treatment were considered together, the weight of the larvae was dependent with a high degree of significance on the concentration of the ecdysteroid agonist ($P < 0.0001$), the length of treatment ($P < 0.0001$), and the choice of JH agonist ($P < 0.0001$), but not which ecdysteroid agonist ($P < 0.5854$) was employed.

Effects of RH 5992 and RH 2485 on Mortality

Mortality for the larvae treated with either RH-5992 or RH-2485 increased above control levels at concentrations of 5.0 ppm or higher, with virtually all of the larvae dying prior to metamorphosis when placed on diet with an ecdysteroid agonist at a concentration of 25 ppm (Fig. 3A and B). An analysis of the mortality data for RH-5992 and RH-2485 averaged over all of the concentrations showed that although the effects of the two ecdysteroid agonists could not be distinguished statistically ($P < 0.2750$), the percent mortality depended on the concentration of either RH-5992 or RH-2485 with a high degree of significance ($R^2 = 0.91$ and 0.70; $P < 0.0001$).

Cumulative mortality of the larvae treated with JH agonist was assessed during a 31-day observation period. When methoprene (10 ppm) or fenoxycarb (5 ppm) was applied simultaneously with RH-5992 or RH-2485 at concentrations of ≤1.0 ppm, mortality of the larvae was in the range of 10–30%, while at an ecdysteroid agonist concentration of 25 ppm the mortality was nearly 100 % (Fig. 3A and B). Interestingly, at intermediate concentrations of 5 and 10 ppm of either ecdysteroid agonist, the simultaneous presence of methoprene or fenoxycarb resulted in increased mortality. For example, there was 44% mortality with 10 ppm of RH-2485 alone, but 100% mortality when either methoprene or fenoxycarb were also present. In addition, we observed that all larvae treated with 25 ppm RH-5992 and fenoxycarb died by day 3, while larvae exposed to RH-5992 alone were still alive on day 3. Similarly, all of the larvae died by day 3 when treated with 25 ppm RH-2485 and methoprene, but not with RH-2485 alone. In general, the presence of a juvenoid compound resulted in the ecdysteroid agonists causing a greater lethality and at an earlier time than otherwise. Thus, while the mortality of the treated larvae increased with the concentration of ecdysteroid agonist, intermediate doses of the ecdysteroid agonists were even more lethal in the presence of either methoprene or fenoxycarb. The dependence of mortality of the larvae on the presence of methoprene on the concentration of RH-5992 or RH-2485 was highly significant ($R^2 = 0.95$ and 0.91, respectively; $P < 0.001$ for each). Mortality in the presence of fenoxycarb was also dependent upon the concentration of either RH-5992 or RH-2485 ($R^2 = 0.91$ and 0.94, respectively, $P < 0.001$ for each). When the combined data for these experiments were analyzed together to test for hormonal interactions, the results demonstrated that the effects of the JH agonists on mortality depended significantly on which ecdysteroid agonist was applied simultaneously ($P < 0.0045$).

Glucosamine Uptake in Tissue Culture

The increased mortality of larvae exposed to ecdysteroid agonists when presented in combination with a juvenoid mimic suggested that there might be an interaction of these compounds that adversely affects the molting process. In an earlier approach to examining the effects of ecdysteroid agonists, we noted that RH-5849 increased ^{14}C- GlcNAc in the PID2 imaginal disc cell line (Silhacek et al, 1990). Given our results with the newer compounds, we tested for influence of RH-5992 and fenoxycarb on ^{14}C- GlcNAc uptake in the PID2 cells, a process that is linked to the incorporation of this chitin precursor into cuticle in intact imaginal discs. In the present experiments, as little as 0.005 μM RH-5992 increased uptake of the isotope by 30% over the untreated control, while fenoxycarb alone decreased uptake by 27%. Moreover, when fenoxycarb was added to the cell cultures simultaneously with RH-5992 there was a 54% decrease in uptake of the isotope compared with RH-5992 alone.

DISCUSSION

Our results demonstrate that the ecdysteroid agonists, RH-5992 and RH-2485, were more effective than RH-5849 in preventing larval growth and in inducing mortality of treated *P. interpunctella* larvae (Silhacek et al., 1990). In the present experiments, larvae reared on diet treated

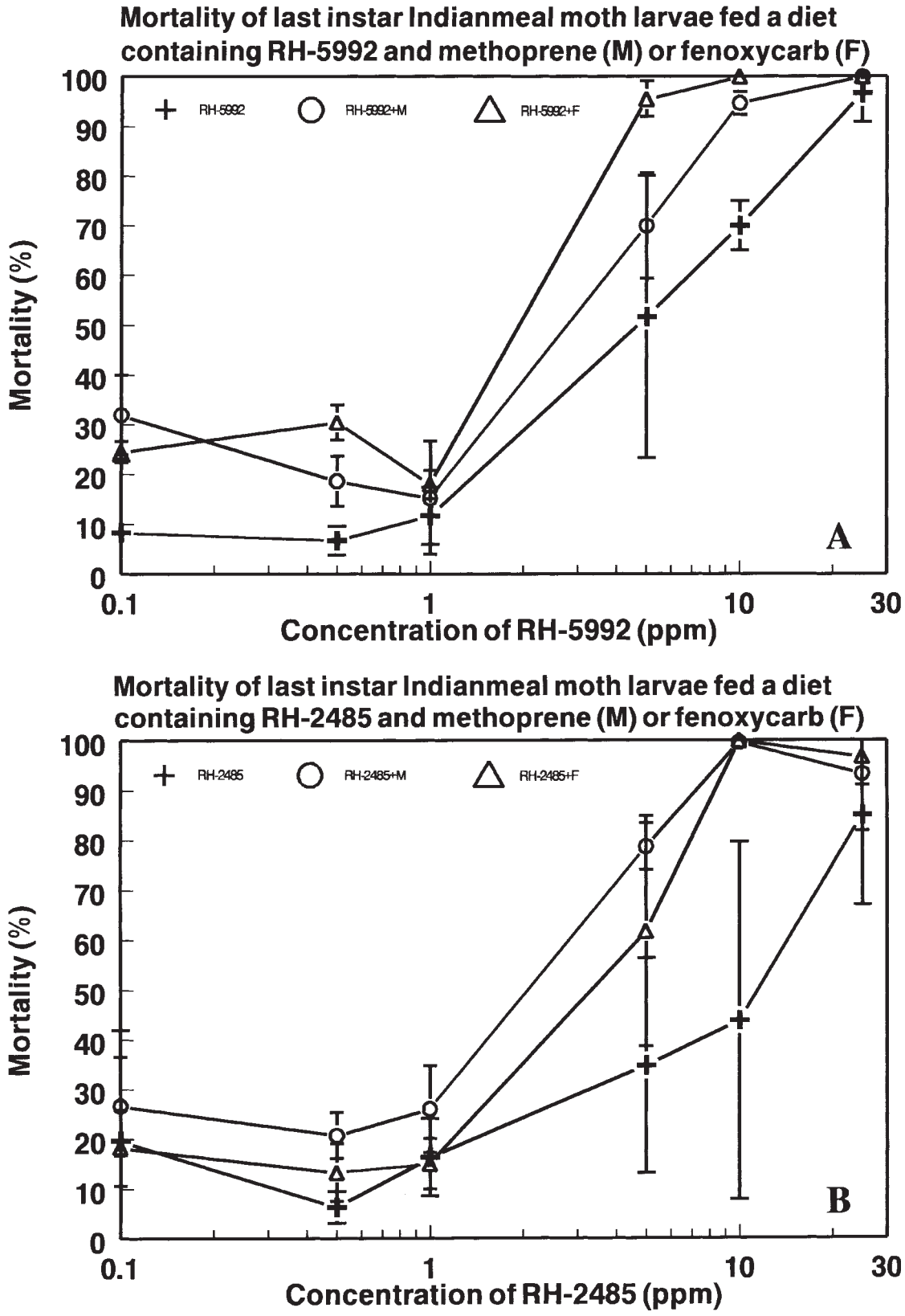

Fig. 3. Mortality (% =/- S.E.) of last instar Indianmeal moth larvae fed a diet of RH-5992 (**A**) or RH-2485 (**B**) with or without methoprene (10 ppm) or fenoxycarb (5 ppm).

with either ≥5 ppm RH-5992 or RH-2485 weighed less in proportion to the dose of ecdysteroid mimic, when compared with larvae reared on untreated diet. Similarly, mortality in untreated larvae was 0–10%, but was as much as 90–100% in larvae reared on diet treated with an ecdysteroid agonist. In these studies, the effects of the two ecdysteroid agonists could not be distinguished, but the results were dependent on the concentration of agonist and on the duration of the treatment. The mortality data for RH-5992, alone, are consistent with the report of Smagghe et al. (1996a) for *P. interpunctella*.

The JH mimics, methoprene and fenoxycarb, not only prevented metamorphosis, but the larvae continued to feed and gain weight throughout the month-long observation period, despite the simultaneous presence of an ecdysteroid agonist. Interestingly, although the addition of JH mimics counteracted the inhibitory effects of the ecdysteroid agonists on growth by causing increased larval weight, they did not prevent mortality. In fact, combinations of a JH mimic with 10 ppm RH 2485 or RH 5992 resulted in nearly 100% mortality compared with 40–70% mortality without the JH compounds, an interactive effect not seen in our earlier tests with RH-5849 (Silhacek et al., 1990). Moreover, the effects of JH agonist on mortality depended on which ecdysteroid agonist was applied in combination. Thus, our results indicate that even though JH mimics overcome the inhibitory effect on weight gain of ecdysteroid agonists, they ultimately caused increased mortality when JH and ecdysteroid agonists were applied in combination.

However, the JH agonists cannot simply be working antagonistically to the ecdysteroid mimics and countering their action. First of all, the JH agonists have the property of preventing metamorphosis, an effect not demonstrated by the ecdysteroid agonists in these studies. Secondly, the extended larval life of test insects treated with JH agonists did not make them immune to the mortality caused by high doses of ecdysteroid agonists, and in fact we observed that moderate doses of ecdysteroid agonist were more lethal when combined with a JH agonist. While the mechanisms that underlay our observations are not apparent, it is clear that the larvae in these studies have been stressed, and in opposing directions. On the one hand, the ecdysteroid agonists acted as a stimulus to the larvae to cease feeding and pupate, while on the other hand, the JH agonists stimulated continued feeding and blocked metamorphosis. The JH agonists, by extending larval life, extended the span of time available for the ecdysteroid agonists to attempt to induce molting, which most often leads to an extra larval molt and death (Oberlander et al., 1995). Moreover, Smagghe et al. (1996b) showed that tebufenozide caused a disruption in the formation of a complete cuticle in treated larval *P. interpunctella*, with a concomitant reduction in endocuticular lamellae that are part of normal cuticle development. This effect on cuticle formation is similar to effects observed in this species with high doses of 20-hydroxyecdysone (Dutkowski et al., 1977). One possible explanation for the synergistic effects of ecdysteroid and juvenoid agonists with respect to mortality may lie in the inhibitory effects of fenoxycarb on ecdysteroid-stimulated uptake of GlcNAc, a chitin precursor.

One strategy to combat resistance of insects to insecticides is to employ combinations of chemicals with different modes of action (e.g., see Oberlander, 1996). Our results point to the possible utility of combining IGRs that mimic both juvenile hormone and ecdysteroids for greater long-term effectiveness in controlling insect pests.

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