Hormonal Insecticides

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Background:

North America has experienced a persistent population of foreign destructive insects. With no natural predators to manage alien insect populations, they can become a serious problem. An example of such an insect is the dipteran European Crane fly, *Tipula paludosa*. Newly hatched larvae feed on root crowns of clovers and grass, causing severe damage to lawns, flowers, vegetables, and small fruits [1]. An attractive pest management strategy is to disrupt the normal insect development, which includes molting and metamorphosis controlled by the steroid hormone 20-hydroxyecdysone (20E). The non-steroidal ecdysone agonists, RH-5992 and RH-5849 are similar to 20E, except that they irreversibly bind to the ecdysone receptors to cause a precocious and incomplete molting that is lethal to larvae [2]. RH-5992 is most effective in lepidopterans, while RH-5849 is most effective in dipterans [3]. Here, the effect of the agonist RH-5849 on the expression of EcR, was observed in the model dipteran; *Drosophila melanogaster*. The effect of RH-5992 was also observed to verify its lack of specificity to *D. melanogaster*.

Purpose:

The purpose of this project is to evaluate a specific hormone analog as potential environmentally friendly "insecticide" for the European Crane fly, *Tipula paludosa*.

Hypothesis:

The presence of ecdysone agonist RH-5849 is expected to continuously up-regulate the level of EcR and thus cause a significant amount of mortality in *D. melanogaster*.

Procedure:

The wild-type strain of *D. melanogaster* was obtained from Boreal Laboratories Ltd. (St. Catharines, Ontario) and reared in white medium. The 20E was purchased from Sigma-Aldrich, and the ecdysone agonists (RH-5992, RH-5849) were generously provided by Dr. Peter J. Krell of the University of Guelph. The 20E and the RH compounds were dissolved in 70% ethanol to 4 mM as stocks.

Dose Dependent Assay:

First instar *D. melanogaster* larvae were treated by feeding with 5 ìM, 7 ìM, and 9 ìM of 20E and the ecdysone agonists RH-5992, RH-5849 mixed in food for 12 hours. Meanwhile, larvae were fed with food containing 70% ethanol and used as a control. Protein extracts were then prepared from whole larvae by homogenizing the frozen larvae in a lysis buffer composed of 20 mM phosphate, pH 7.8, 300 mM NaCl, 1% NP-40, 0.2 mM phenylthiourea, and a cocktail of serine and cysteine-type protease inhibitors. The homogenate was then centrifuged at 5000 × g for 15 minutes [4]. The protein concentration of supernatants was determined by the Bradford method [5]. A 10% SDS-PAGE gel was performed, followed by Coomassie blue staining. BenchMark pre-stained protein ladder (Invitrogen) was loaded onto the gel to estimate the molecular mass of the EcR protein.

Time Course Study:

To examine the biological effects of 20E and RH-5849, first instar *D. melanogaster* larvae were treated with 9 µM 20E or RH-5849 the same way as described above for 24, 48, and 96 hours at room temperature with 30 larvae per experimental condition. At each time point, samples were examined under a dissecting microscope. The number of larvae in each instar, as

well as the number of deaths at each time point, were recorded. For studies at the molecular level, a time course experiment was performed using time points of 6, 12, 24, 48, and 96 hours. First instar *D. melanogaster* larvae were treated with 9 µM of 20E or RH-5849. The protein composition was analyzed using SDS-PAGE and Coomassie-blue staining. The expected position of the EcR band was estimated based on its molecular mass, using a standard curve generated from the pre-stained protein marker. The relative amount of EcR protein per total protein was quantified using FluorChem 8900 (Alph-Innotech) and calculated using the integrated density value (IDV).

Results and Conclusions:

The dose dependent assay revealed that while all doses of RH-5849 up-regulated the level of EcR protein, treatments with RH-5992 had no significant effect on the EcR protein level (data not shown). This confirms the specificity of RH-5849 discussed in earlier publications [6]. This is crucial to the development of an environmentally friendly insecticide since it should be target specific.

In the 96-hour time course experiment the ecdysone agonist RH-5849 successfully sped up the *D. melanogaster* growth cycle, mimicking the ecdysone hormone 20E as measured by percentage of larvae in different stages of development. However, unlike 20E, treatment of larvae with RH-5849 resulted in a significant percentage of deaths (Fig. 1). Clearly, treatment with RH-5849 resulted in a higher mortality rate, an essential property of a pest management agent. This was also reflected at the molecular level, where the EcR level was consistently up regulated in the RH-5849 treatment (Fig. 2)

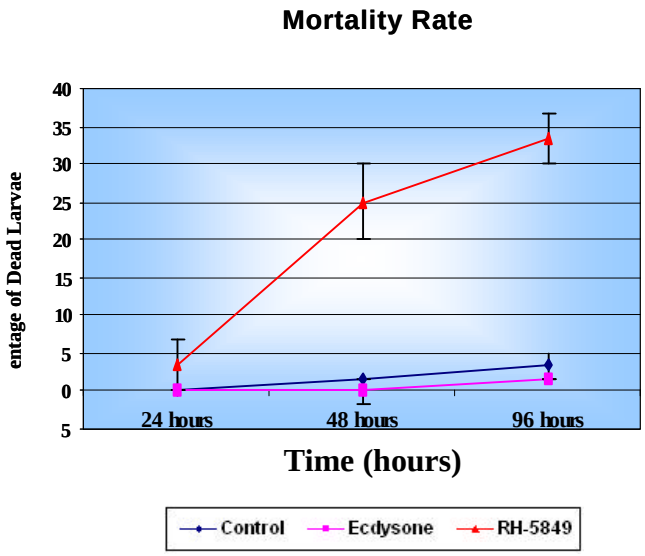

Figure 1. Mortality rate of the control, ecdysone, and RH-5849 samples

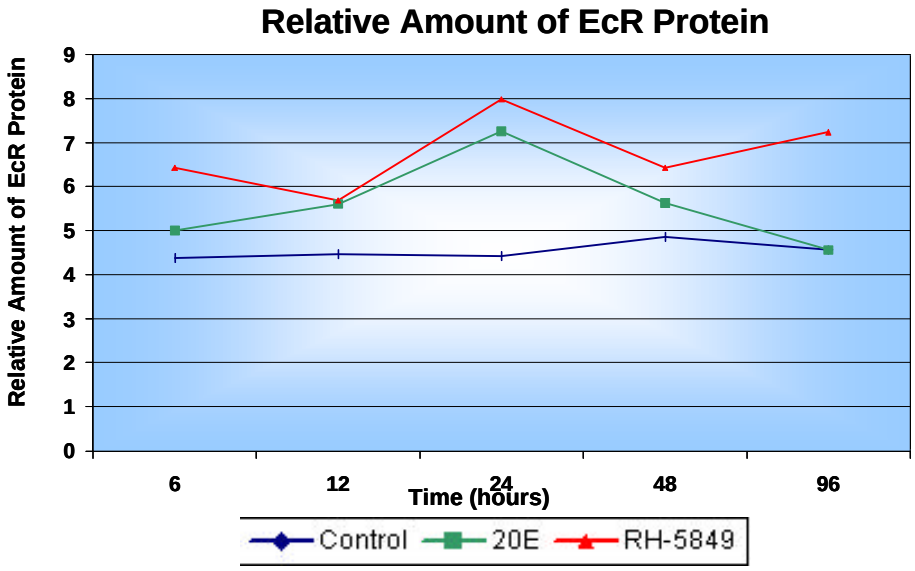

Figure 2. Changes of the relative amount of EcR protein at various times after treatment with 20E or RH-5849 as compared to the control

As illustrated in Fig. 2 above, there was a significant difference between the relative amount of EcR protein in the control, 20E, and RH-5849 treatments. Throughout the time course in the control treatments, the level of EcR remained relatively static. The samples treated with 20E experienced an up-regulation of EcR from 6 to 24 hours and a down-regulation of EcR from 24 to 96 hours. This suggested that 20E ceases to stimulate the EcR protein after 24 hours, enabling complete molting to occur. Unlike 20E, RH-5849 treated samples had an overall

increase in the amount of EcR from 6 to 96 hours. This implied that RH-5849 binds irreversibly to the EcR protein. This would result in continuous stimulation of the ecdysone pathway and prohibiting other proteins from being expressed resulting in incomplete and lethal molts in *D. melanogaster*. To further explore the effects of RH-5849, tests using reverse transcriptase-PCR (RT-PCR) will be performed to evaluate the levels of EcR mRNA under different experimental conditions in the near future.

Acknowledgements:

We thank Dr. Peter J. Krell and his assistants at the University of Guelph for their mentorship, support, and for providing lab space and equipment. We also thank our teacher at Centennial C.V.I., Mr. Doug Gajic, for his support and guidance.

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APPENDIX 1

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