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Review

Stage-specific action of juvenile hormone analogs

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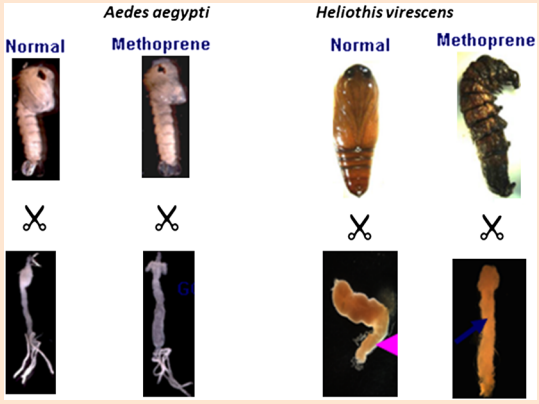
The discovery of juvenile hormones (JH) and their synthetic analogs (JHA) generated excitement and hope that these compounds will replace first- and second-generation insecticides that have not so desirable environmental and human safety profiles. However, JHAs used commercially during the past four decades did not meet these expectations. The recent availability of advanced molecular and histological methods and the discovery of key players involved in JH action provided some insights into the functioning of JHA in a stage and species-specific manner. In this review, we will summarize recent findings and stage-specific action of JHA, focusing on three commercially used JHA, methoprene, hydroprene and pyriproxyfen and economically important pests, the red flour beetle, *Tribolium castaneum*, and the tobacco budworm, *Heliothis virescens*, and disease vector, the yellow fever mosquito, *Aedes aegypti*.



Keywords: methoprene, hydroprene, pyriproxyfen, pest and vector.

Introduction

The discovery of juvenile hormones (JH) that control insect development to keep them in the juvenile stage by preventing metamorphosis and chemical analogs that mimic JH action caused tremendous excitement in the insect control industry.[1–7] The JH analogs (JHA) were framed as "third-generation insecticides" because of their presumed insect-specific action due to the absence of juvenile hormones in most non-arthropod animal species. These compounds were considered much safer to humans, animals and the environment when compared to first generation insecticides (inorganic compounds such as sulfur, arsenic, hydrogen cyanide, mercury, lead) and second-generation insecticides (synthetic compounds such as DDT, carbamates, organophosphates), which cause major non-target effects on human and animal health and other members of the ecosystem. The World Health Organization recommends the use of methoprene treatment of water near dwellings for mosquito control. JHA, methoprene, was registered as a biological pesticide by the USA EPA in 1975 but was re-classified later as a biochemical pesticide.

To the disappointment of JHA proponents as an answer to pest control problems, JHA did not live up to the expectations. JHAs have been a huge disappointment in controlling pests that damage crops, orchards, and forest trees, especially those inflicting damage during immature stages. Limited success has been achieved in controlling crop pests that cause damage during adult stages, flies, fleas, mosquitoes, and other insects that transmit human and animal diseases during adult stages. Studies over the years showed that the sensitivity and effectiveness of JHA vary quite a bit among insects and even among life stages of the same insect.[8] Recent advances in histology, microscopy, molecular, genetic, genome sequencing, and functional genomics methods allowed for increased understanding of differences in JHA mode of action in different insect species and different stages in each insect species. This information could help pest control operators in choosing JHA for controlling insect pests,

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medically important insects, and disease vectors. This information also could be used for deciding the timing of the application of JHA. In this review, we will focus on describing recent studies that demonstrated similarities and differences in mode of action of the three most used JHA, methoprene, pyriproxyfen and hydroprene, among insects studied. We will focus on the mode of action in immature insects and cover studies in economically important insects, including the yellow fever mosquito, *Aedes aegypti* (Diptera), the red flour beetle, *Tribolium castaneum* (Coleoptera), the silk moth, *Bombyx mori* and the tobacco budworm, *Heliothis virescens* (Lepidoptera).

1. JHA application induces larval period extension in most insects

As with most other insecticides, the application of JHA to the appropriate stage of insects is particularly important to achieve effective control of target insects that destroy food, fiber, forests and transmit diseases. Several studies, as described below, illustrated the stage- and insect species-specific effects of JHA. In most insects, JHA application during early larval stages induces supernumerary molts, but application during the last instar stage often fails to induce extra larval molts but interferes with metamorphosis. For example, one to three extra molts were observed in the turnip aphid treated with JHA, pyriproxyfen during the first three instars. However, the treatment of fourth (last) instar nymphs with the same JHA did not induce extra molts.[9] Similar effects were reported for pyriproxyfen treatment of soybean aphid.[10] In Asian citrus psyllid, *Diaphorina citri* methoprene treatment blocks 95% adult emergence when treated during the first three instars, and only 60% block in adult emergence was detected when treated during the last instar stage.[11] In some insects such as the German cockroach, *Blatella germanica*, treatment with pyriproxyfen during the first 14 days of the fifth and last instar blocks metamorphosis and produces giant nymphs.[12] In most lepidopteran insects, the application of JHA to early last instars induces the development of supernumerary instars, whereas application of JHA during the final instar stage results in abnormal pupation and development of larval–pupal mosaics or intermediates. Application of JHA, fenoxycarb, during the larval stages, induced an extra larval molt or larval–pupal intermediates as observed in the silkmoth, *B. mori*.[13] In the swallowtail butterfly, *Papilio demoleus*, JHAs pyriproxyfen and diofenolan, induced similar effects as in other lepidopteran insects in prolonging larval stages.[14,15] In lepidopteran insects, JHA often prolongs the last larval stage resulting in additional feeding and damage of crop plants or stored products. This is one of the reasons for preventing the wide-spread use of JHA in controlling lepidopteran pests. In both *H. virescens* and *T. castaneum*, application of JHA methoprene and hydroprene, respectively, induce the extension of larval stage and defects in larval development as reported in insects from other orders. The effect of JHA application on these two insects will be discussed in detail in the following sections.

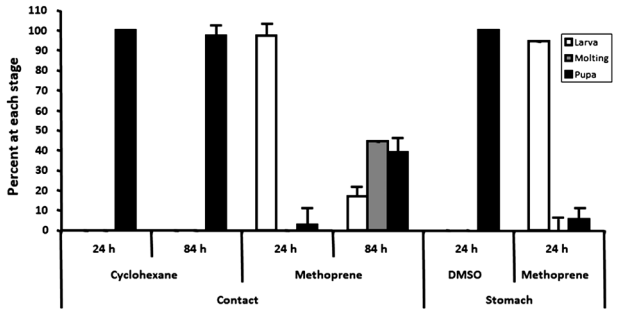

Fig. 1. Effect of methoprene on the development of *Heliothis virescens*. Cyclohexane or methoprene in cyclohexane at 20 μg/larva was applied topically at 24 hr and at 84 hr after ecdysis into the final larval stage of *H. virescens*. Oral treatment was done by feeding larvae on DMSO or methoprene containing diet beginning at 24 hr after molting into last instar larval stage. Twelve larvae were used in each treatment and the treatments were replicated three times. Mean $\pm$ S.D. for the three independent experiments are shown.

1.1. Heliothis virescens

Application of methoprene dissolved in cyclohexane on *H. virescens* integument at 24 hr after ecdysis into the final larval stage blocked metamorphosis; 98% of the treated insects remained in the larval stage (Fig. 1). In contrast, application of methoprene at 84 hr after ecdysis into the final instar larval stage (after commitment to become pupae) blocked larval–pupal metamorphosis in only 20% of the treated insects. Among the rest of the treated insect, 40% formed larval–pupal intermediaries while the remaining 40% successfully pupated, but pupae were malformed and died during the pupal stage. Cyclohexane alone did not cause any effect (Fig. 1). To determine whether route of application makes any difference, *H. virescens* larvae were fed on diet containing methoprene dissolved in DMSO or DMSO alone beginning at 24 hr after ecdysis into the final instar larval stage. As shown in Fig. 1, all the larvae fed on DMSO containing diet successfully pupated. In contrast, more than 90% of the larvae fed on methoprene containing diet remained in the larval stage. These data confirm previous reports about effect of JHA in lepidopteran insects. Applications prior to commitment prolong larval period while application after commitment to larval–pupal metamorphosis cause variable effects, including the formation of larval–pupal intermediates and defects and death of pupae developed from JHA treated larvae. Unlike in *Ae. aegypti* and other dipteran insects, the JHA applied prior to committing to the larval–pupal metamorphosis of lepidopteran insects such as *H. virescens* blocks metamorphosis. We hypothesized that these differences in JHA effects between lepidopteran and dipteran insects might be due to differences in expression of genes involved in 20-hydroxyecdysone (20E) regulation of metamorphosis. To test this hypothesis, we determined mRNA levels of ecdysone receptor (EcR) and ultraspiracle (USP) in midgut and epidermis dissected from staged *H. virescens* final instar larvae treated with methoprene or solvent. The mRNA levels of EcR and USP increased during the prepupal stages in both midgut and epidermis (Fig. 2). Application of methoprene during the early

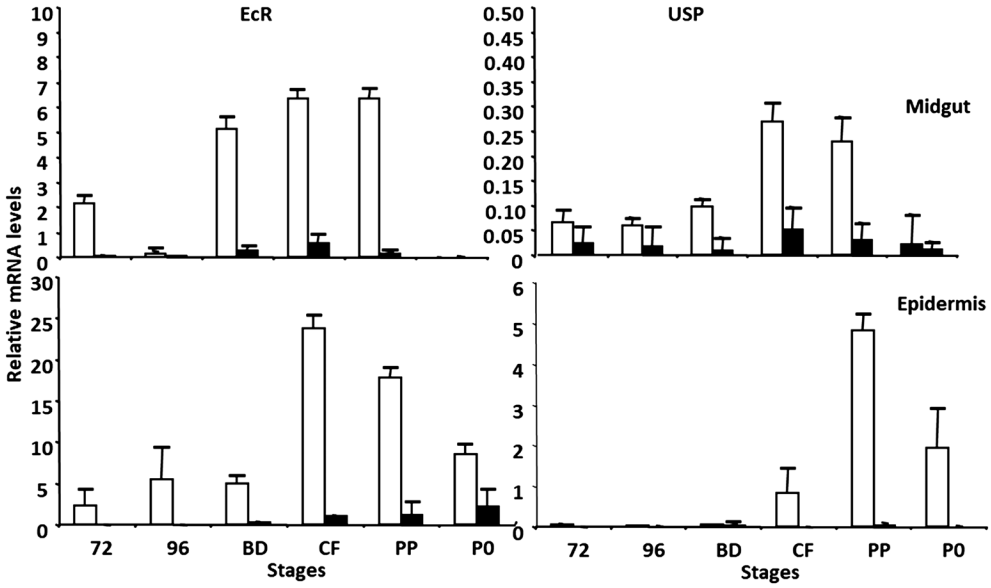

Fig. 2. Relative mRNA levels of ecdysone receptor (EcR) and ultraspiracle (USP) in the midgut and epidermis isolated from methoprene-treated and untreated *H. virescens* larvae. The mRNA levels were quantified using RT-qPCR. mRNA levels were normalized using ribosomal RNA as a standard. Mean relative expression ± S.E. for three independently staged sets of final instar larvae (72–96 hr) and pupae are shown. BD-burrowing and digging stage, CF-cell formation stage, PP—prepupal stage, P0—white pupal stage.

final instar larval stage prevented this increase in EcR and USP mRNA levels in both midgut and epidermis. These data suggest that methoprene affected the expression of EcR and USP in a similar way in both the midgut and epidermis, resulting in larval stage extension and block in metamorphosis to the pupal stage.

1.2. Tribolium castaneum

In the coleopteran insect, *T. castaneum*, supernumerary larval molts were induced in larvae treated with hydroprene.[16] However, the differential response was detected based on the time method of application. Hydroprene fed to larvae at different time points during the larval stage showed differential effects. More than 90% of the larvae fed on hydroprene during the penultimate or until 60 hr after ecdysis into the final instar remained in the larval stage and molted to the supernumerary larval stage. However, feeding hydroprene beginning at 72 or 96 hr after ecdysis into the final instar did not block larval–pupal metamorphosis as more than 90% of the treated larvae completed larval stage and larval–pupal metamorphosis. However, the pupae developed from hydroprene treated larvae died during the pupal stage. The topical application of hydroprene during the last instar larvae showed phenotypes different from these observed in hydroprene fed larvae. Application of hydroprene to larvae at 72 hr after ecdysis into the final instar induced mortality of 100% of treated larvae during the quiescent stage. In contrast, hydroprene treatment at 84 hr after ecdysis into the final instar induced larval–pupal intermediaries. The pupae developed from these larvae showed defects in the development of pupal structures, including wings. Hydroprene treatment of larvae at 96 hr after ecdysis into the final instar did not block larval–pupal metamorphosis: all the larvae pupated. However, the pupae developed from these larvae showed defects in pupal structures and died during the pupal stage. These data from *H. virescens* and *T. castaneum* as well other reports from fire ant; *Solenopsis invicta*,[17] the bark beetle; *Ips paraconfusus*[18]; and the tobacco cutworm, *Spodoptera litura*[19] suggest that JHA effects vary with the dose, time, and the method application. Therefore, for maximum efficacy of JHA, one needs to pay attention to the stage of the target insect and methods of application of insecticide.

2. JHA application kills pupae but not larvae in some insects

As explained above, in most insects, application of JHA prior to commitment to metamorphosis blocks metamorphosis and prolongs the duration of immature stages.[20] In contrast, continuous exposure of newly molted *Ae. aegypti* 3rd instar larvae, final instar larvae, 48 hr-old final instar larvae to methoprene does not block larval development or larval–pupal metamorphosis. The treated larvae develop to the pupal stage and die as pupa (Fig. 3). Previous studies showed that exposure of *Ae. aegypti* larvae to JHA throughout larval life did not block larval–pupal metamorphosis.[21] The methoprene treated larvae successfully pupated and died during the prepupal stage. Braga *et al.*, 2005[22] also showed that *Ae. aegypti* exposed to methoprene during larval stages successfully pupated and died during the pupal stage and the mortality is methoprene dose-dependent. Interestingly, methoprene is effective in killing temephos-resistant Brazilian *Ae. aegypti* populations by methoprene application alone, suggesting that JHA could substitute for temephos for controlling this insect vector.[23] JHA pyriproxyfen inhibits adult emergence of Australian salt-marsh mosquito, *Aedes vigilax*.[24] Pyriproxyfen was shown to inhibit *Aedes japonicus* adult emergence after exposure of 3rd or 4th instar larvae to this insecticide. Addition-

ally, gravid adult females were used to auto-disseminate pyriproxyfen powder to larval development habitats to inhibit adult emergence.[25] Many studies showed the effectiveness of pyriproxyfen dissemination through gravid adult females to reach cryptic larval habitats for their control and inhibition of adult emergence.[26,27] These studies showed that pyriproxyfen has immense potential for use in controlling this mosquito. EcoBio-Block S, a novel controlled release system of JHA pyriproxyfen, inhibited adult emergence of *Aedes* mosquitoes.[28] Since many species of adult mosquitoes transmit diseases, preventing adult emergence using JHA such as methoprene and pyriproxyfen has been an extraordinarily successful weapon in preventing the spread of infectious diseases. Methoprene has been used to control mosquitoes for many years.[23,29–31] The use of JHA pyriproxyfen for controlling *Aedes* mosquitoes has increased dramatically during the past few years,[32,33] a recent review summarizes these applications.[34]

3. Application of JH blocks death of larval cells, but the effect on the proliferation and differentiation of imaginal cells is variable

3.1. Heliothis virescens

In *H. virescens* larvae treated with methoprene, programmed cell death (PCD) in midgut larval cells is blocked.[20] Methoprene application results in an increase in expression of the gene coding for the inhibitor of apoptosis (IAP) and a decrease in expression of genes coding for caspase-1, ICE, and caspase-3 protein levels. The proliferation and differentiation of imaginal cells were affected by methoprene treatment.[20] These studies demonstrate that application of JHA during that final instar larval stage influences remodeling of larval tissues and development of pupal tissues leading to the formation of larval/pupal deformities.

3.2. Tribolium castaneum

Application of hydroprene during the final instar larval stage affected both programmed cell death (PCD) of larval cells and proliferation and differentiation of imaginal cells to pupal gut epithelium were impaired.[16] Hydroprene suppressed the expression of EcRA, EcRB, Broad, E74, E75A, and E75B, resulting in a block in midgut remodeling.

3.3. Aedes aegypti

In normal *Ae. aegypti* larvae grown at 23°C, the PCD of larval midgut cells and the proliferation and differentiation of imaginal cells start at 36 hr after ecdysis to the 4th instar larval stage and completed by 12 hr after ecdysis to the pupal stage. In larvae exposed to methoprene continuously during the larval stage, the proliferation and differentiation of imaginal cells were initiated at the normal time, but the PCD was initiated only after ecdysis to the pupal stage and the elimination of larval midgut was not completed.[21] As a result, the pupae developed from the methoprene treated larvae contain both larval and pupal midguts and die during the pupal stage. The expression of genes coding for proteins involved in 20E action (EcRB, USPA, broad complex, E93 and ftz-f1) and programmed cell death (dronc and drice) was affected by methoprene treatment.[21] Thus, in *Ae. aegypti* and in other dipteran insects, JHA application blocks midgut remodeling, but the synthesis of pupal cuticle and ecdysis to the pupal stage are not affected by methoprene treatment. As a result, no matter when JHA is applied to these insects, they will die during the pupal stage.

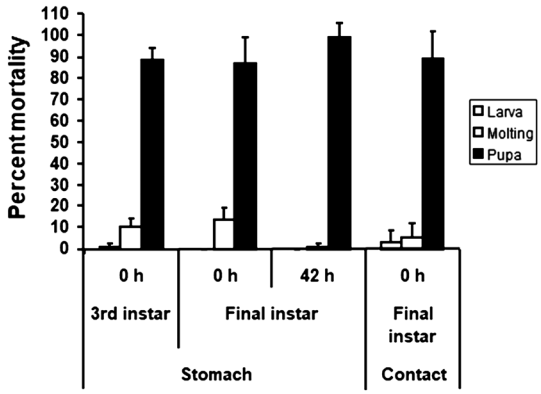

Fig. 3. Effect of methoprene on the development of *Aedes aegypti*. *Ae. aegypti* were treated with methoprene by transferring larvae at the beginning of 3rd and final instar larval stage into water containing 50 ng/mL methoprene in DMSO or DMSO. Cyclohexane or methoprene in cyclohexane at 100 ng/larva was also applied topically at the beginning of the final instar larval stage. Ten larvae were used in each treatment and the treatments were replicated three times. Mean±S.D. for the three independent experiments are shown.

4. JHA efficacy is variable among insect pests and disease vectors tested

The efficacy of the three most used JHA, methoprene, hydroprene and pyriproxyfen for controlling pests and insects of medical importance seems vary among the species of insects tested. Methoprene is the most widely used JHA for controlling mosquito larvae.[22,23,29–31,35–45] However, recent studies showed the efficacy of pyriproxyfen for larval control and adult sterilization of various mosquito species,[24,28,32–34,46–66] which might increase the use of this chemical for vector control. In contrast, hydroprene was not used much for mosquito control. Methoprene is used to control storage pests such as *R. dominica*[67] and *T. castaneum*.[68] Methoprene was also shown to be effective in controlling horn flies on cattle,[69–72] house flies[73] and ticks.[74] Pyriproxyfen was shown to be effective against stored product pests such as *Liposcelis bostrychophila Badonnel, Liposcelis decolor and Liposcelis paeta Pearman*,[75] fleas,[76] tsetse flies[77] and agriculture pests including the greenhouse whitefly, *Trialeurodes vaporariorum* and the sweet potato whitefly, *Bemisia tabaci*.[78–80] Coleopteran insects such as the red flour beetle, *T. castaneum* is highly susceptible to hydroprene.[81] Hydroprene is used to control stored product pests,[82,83] cockroaches[84–86] and bed bugs.[87]

Conclusions and future prospective

The JHAs have been commercially used in the USA and other

countries around the globe for 45 years. Although JHAs did not meet the initial expectations, these insecticides found their niche market for controlling adult pests and disease vectors. Methoprene and pyriproxyfen are currently used in large quantities to control mosquitoes. The use of pyriproxyfen in adult sterilization and control is beginning to pick up. There are hints of resistance development against these compounds[88–90]; this may hinder the wide-spread use of these chemicals in insect control. Research aimed at discovering and developing novel and highly potent JH agonists and antagonists is urgently needed. Armed with JH receptor, target DNA sequences and cell lines that respond to JH very well, the future looks promising for the discovery and development of novel JH agonists and antagonists for controlling pests and disease vectors. Expanding knowledge on JH signaling pathways and advancement in *in silico* predictions of small molecules should enable novel JHA discovery.

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