Effects of juvenile hormone (JH) analog insecticides on larval development and JH esterase activity in two spodopterans

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Juvenile hormone analog (JHA) insecticides are biological and structural mimics of JH, a key insect developmental hormone. Toxic and anti-developmental effects of the JHA insecticides methoprene, fenoxycarb, and pyriproxyfen were investigated on the larval and pupal stages of Spodoptera littoralis and Spodoptera frugiperda. Bioassays showed that fenoxycarb has the highest toxicity and fastest speed of kill in 2nd instar S. littoralis. All three JHAs affected the development of 6th instar (i.e., final instar) and pupal S. frugiperda. JH esterase (JHE) is a critical enzyme that helps to regulate JH levels during insect development. JHE activity in the last instar S. littoralis and S. frugiperda was 11 and 23 nmol min⁻¹ ml⁻¹ hemolymph, respectively. Methoprene and pyriproxyfen showed poor inhibition of JHE activity from these insects, whereas fenoxycarb showed stronger inhibition. The inhibitory activity of fenoxycarb, however, was more than 1000-fold lower than that of OTFP, a highly potent inhibitor of JHES. Surprisingly, topical application of methoprene, fenoxycarb or pyriproxyfen on 6th instars of S. littoralis and S. frugiperda prevented the dramatic reduction in JHE activity that was found in control insects. Our findings suggest that JHAs may function as JH agonists that play a disruptive role or a hormonal replacement role in S. littoralis and S. frugiperda.

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1. Introduction

Juvenile hormones (JHs), sesquiterpenoid molecules synthesized by the corpora allata, are critical insect hormones that regulate a large diversity of processes during postembryonic development and adult reproduction in insects [1–3]. Most insects utilize a form of JH known as JH III (epoxy farnesoic acid methyl ester, Fig. 1A) [4]. Lepidopteran insects have additional JH molecules termed JH 0, JH I, and JH II that have ethyl substitutions of the methyl side chains of JH III [5]. JHs along with ecdysteroids (i.e., molting hormones) play key roles in the molting process. Simplistically, high JH titer maintains the “status quo” action which modulates a larval–larval molt, while a low-to-absent JH titer allows ecdysone (molting hormone) to direct a more developmentally advanced molt [6]. JHs are metabolized in lepidopterous larvae and other juvenile insects by a JH esterase (JHE) and a JH epoxide hydrolase (JHEH) [7,8]. The methyl ester of JH is hydrolyzed by JHE, while the epoxide group is hydrolyzed by JHEH. Both JHE and JHEH belong to the α/β-hydrolase fold superfamily, and generate JH acid and JH diol, respectively, from JH. JHEs show unique biochemical (e.g., high specificity (Kcat/KM ratio) for JH), structural, and biological characteristics that help to differentiate JHEs from other esterases that are found in the hemolymph [9].

JH analog (JHA) insecticides are novel and effective green compounds that target the endocrine system of pest insects but appear to lack vertebrate toxicity [10]. JHA insecticides, compounds that mimic JH structure and/or its biological action, were developed based on the identification of molecular targets that are specific to insects and used for disrupting the insect life cycle [6]. Although it is clear that JHAs act as JH agonists once inside an insect, it is still unclear how these compounds kill insects. Experimentally, the application of JHA insecticides during the egg, pupal or adult stage results in the failure of egg hatch for many species [11,12] probably due to the disruption of early embryonic development; mortality during pupal development; and sterility in some adult species, respectively [10,12]. Larvae generally are poorly susceptible to JHA treatment since endogenous JH is already high. Mechanistically, JHAs have been hypothesized to function as inhibitors of JHE or possibly JHEH [13]. These enzymes are required to reduce JH titer below the status quo level that is important for normal molting to occur. In a manner consistent with this hypothesis, the topical application of 3-octylthio-1,1,1-trifluoro-2-propanone (OTFP), a slow tight binding inhibitor of esterases [14], causes developmental defects in insects that are similar to that observed following the application of JHA insecticides [15]. In this study, we investigate the in vivo effects of the topical application of the JHA insecticides methoprene, fenoxycarb, and pyriproxyfen on larval and pupal Spodoptera littoralis.
and *Spodoptera frugiperda*. Subsequently, we investigate the ability of these JHAs to inhibit JHE activity both in *vivo* and in the hemolymph following topical application of 6th instar larvae. We found that fenoxycarb shows the highest inhibitory potency of the three JHAs that were tested; however, this inhibitory activity was significantly lower than that of OTFP. Interestingly, all of the JHA insecticides that were tested in *vivo* prevented the dramatic reduction in JHE activity that was found in control insects.

2. Materials and methods

2.1. Rearing of *S. littoralis* and *S. frugiperda*

The eggs of *S. littoralis* were obtained from Agriculture Research Center (ARC) (Giza, Egypt), and the eggs of *S. frugiperda* were obtained from Benzon Research (Carlisle, PA). The larvae were reared on a premixed artificial diet (Bioserv#F9781B, Frenchtown, NJ). The larvae were main- 
tained at 26 ± 2 °C on a 14:10 (L:D) photoperiod.

2.2. Insecticides, substrates, and inhibitors

The JHA insecticides methoprene, fenoxycarb, and pyriproxyfen (all technical grades) were purchased from Sigma-Aldrich (St. Louis, MO) (Fig. 1B, C & D). Unlabeled JH III, and the serine protease/esterase inhibitor diisopropylfluorophosphate (DFP) were purchased from Sigma-Aldrich. Tritium-labeled JH III (11.5 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). 3-Octylthio-1,1,1-trifluoropropan-2-one (OTFP), a slow and tight binding inhibitor of JHE [14] was synthesized in the laboratory as described previously [16].

2.3. Bioassays

The effects of methoprene, fenoxycarb, and pyriproxyfen on insect survival were determined on 2nd instar *S. littoralis* that were allowed to feed on insecticide-contaminated diet mixtures following methods that were previously described [17]. In brief, 5 stock concentrations of each insecticide were prepared in acetone:water (1:1 ratio). 1 ml of each insecticide stock was then mixed with 29 ml of diet in order to generate each insecticide-diet mixture concentration (1 to 100 μg ml⁻¹ diet for methoprene and 1 to 50 μg ml⁻¹ diet for fenoxycarb or pyriproxyfen). 10 ml of each insecticide-diet mixture was then poured into a disposable 9 cm diameter Petri dish. Negative control diet was prepared with acetone:water only (1:1). For each insecticide concentration, groups of 10 2nd instar larvae of *S. littoralis* were tested in triplicate (i.e., 30 larvae/insecticide concentration). Each of these experiments was subsequently repeated two more times on different days. Larval mortality was recorded at 10 days of continuous exposure for each experiment. The larvae were considered dead when they did not respond to a gentle puff of air.

Median lethal time of each JHA insecticide was determined using an LC₅₀ concentration of each insecticide using 2nd instar *S. littoralis*. The insecticide infused diet was prepared as described above. Thirty 2nd instar larvae were continuously exposed to the contaminated diet in triplicate. Control insects were allowed to feed on diet with acetone only as described above. Mortality was recorded daily for 10 days. The time-response experiments were repeated 3 times.

Developmental effects of the JHAs were studied on 6th instar and pupal *S. frugiperda* following topical application of each insecticide. Each insecticide (1 μg of methoprene, fenoxycarb or pyriproxyfen in 2 μl of acetone) was applied on the dorsal surface of thirty larvae or pupae and the phenotype (e.g., normal pupa and adult emergence) of each insect was recorded daily.

2.4. Hemolymph collection and standard JHE assay

Hemolymph from *S. littoralis* or *S. frugiperda* was collected from 6th instar larvae of various masses until the pre-pupal period (groups of three insects per mass range). For *S. littoralis*, larvae which ranged in mass from 400–500, 501–600, 601–700, 701–750, 751–800, 801–850, 851–900, and >900 mg, as well as pre-pupa were used for hemolymph collection. For *S. frugiperda*, larvae which ranged in mass from 400–450, 451–500, 501–550, 551–600, and >600 mg, as well as pre-pupa were used. Hemolymph was collected from each insect by piercing a proleg and holding the insect with gentle pressure over a glass collection tube containing a few crystals of phenylthiourea (PTU) to inhibit tyrosinase activity. Hemolymph samples were diluted 1:5 with assay buffer (AB: 0.1 M sodium phosphate buffer, pH 7.4), containing 0.05% (w/v) Triton X-100, 0.002% (w/v) sodium azide, 1% (w/v) sucrose, 1 mM mercaptoethanol, and 0.005% (w/v) PTU. Diluted hemolymph samples were centrifuged at 1000 × g for 5 min and kept at 4 °C prior to JHE activity determination.

JHE activity was assayed by the JH partition assay method described previously [18]. For each assay, the final concentration of tritium-labeled JH III (New England Nuclear Research Products, Boston, MA: 17 Ci/mmol) and unlabeled JH III (Sigma-Aldrich) was 5 mM in 100 μl of diluted hemolymph. The reaction mixture, in triplicate, was incubated for 15 min at 30 °C. In order to stop the enzyme reaction, 100 μl of basic methanol (methanol:water:ammonium hydroxide, 10:9:1) was added to the stopped reaction in order to partition JH III (isooctane layer) from the JH III acid (aqueous layer). 50 μl of the aqueous layer was mixed with 1.0 ml of ScintiVerse BD Cocktail (Fisher Chemical, Pittsburgh, PA) liquid scintillation counting solution and radioactivity was measured using a Tri-Carb 2810 TR scintillation counter (PerkinElmer). JHE activity was determined in triplicate and expressed as nmol of JH III acid formed min⁻¹ ml⁻¹ hemolymph.

2.5. Inhibition of JHE activity in the hemolymph of *S. littoralis* and *S. frugiperda* by JHAs, OTFP, and DFP

The median inhibitory concentration (IC₅₀) of the JHA insecticides, OTFP, and DFP for hemolymph JHE activity from *S. littoralis* and *S. frugiperda* was determined by modification of the standard JHE assay described above. At least 5 different concentrations of each of the JHAs ranging from 10–1000 mM for methoprene, 0.1–50 mM for fenoxycarb, and 5–250 mM for pyriproxyfen were prepared in ethanol. Six different concentrations of OTFP ranging from 0.05 to 10 μM were prepared in ethanol. For DFP, six different concentrations ranging from 1–300 mM were prepared freshly in ethanol. The diluted hemolymph (100 μl) was preincubated with each of these compounds (1 μl) for 5 min at 30 °C before the addition of the JH III substrate to the reaction
mixture. Following the addition of the JHA or inhibitor, the JHE enzyme reaction was allowed to proceed as described above. LC$_{50}$ values were estimated using SigmaPlot 11 software by regression wizard analysis using a sigmoid 4 parameter setting. The percentage of ethanol in the enzyme reaction mixtures and control was 2%. Each assay was performed in triplicate and repeated three times.

### 2.6. Competitive interaction of JH III and each JHA

The ability of methoprene, fenoxycarb, and pyriproxyfen to competitively interact with JH III for the substrate binding site of JHE from _S. littoralis_ or _S. frugiperda_ was determined by modification of the standard JHE assay described above. The competition assay was performed in a 100 μl reaction volume so that the molar ratio of JH III and each JHA was 1:1 or 1:10, respectively, as described previously.[13] In the 1:1 competition assay, the reaction mixture contained 5 μM JH III, 5 μM JHA, 2% (v/v) ethanol, and diluted hemolymph from _S. littoralis_ or _S. frugiperda_. In the 1:10 competition assay, each reaction mixture contained 0.5 μM JH III, 5 μM JHA, 2% (v/v) ethanol, and diluted hemolymph. In these assays, the JHA (diluted in 100% ethanol) was added to the reaction mixture first then immediately afterwards the JH III was added, and the reaction mixture was incubated at 30 °C for 15 min. The assays were performed in triplicate and repeated three times.

### 2.7. In vivo effect of JHAs on JHE activity from _S. littoralis_ and _S. frugiperda_

The in vivo effect of methoprene, fenoxycarb, and pyriproxyfen on JHE activity in the hemolymph was studied using hemolymph collected from the treated pre-wandering 6th instar _S. littoralis_ (801–900 mg) and _S. frugiperda_ (451–550 mg). The larvae were treated topically using a dose of 1 μg of each JHA in 2 μl of acetone/larva (i.e., 1610 μM, 1659 μM, and 1556 μM of methoprene, fenoxycarb, and pyriproxyfen, respectively, per larva) by application on the dorsal surface. Equal dose of 1:1 competition assay, the reaction mixture contained 5 μM JH III, 5 μM JHA, 2% (v/v) ethanol, and diluted hemolymph. In these assays, the JHA (diluted in 100% ethanol) was added to the reaction mixture first then immediately afterwards the JH III was added, and the reaction mixture was incubated at 30 °C for 15 min. The assays were performed in triplicate and repeated three times.

### 2.8. Statistical analysis

Lethal concentration was determined by Probit analysis[19] using Polo-PC Plus version 3.1 statistical software (LeOra Software, Berkeley, CA) and shown with their 95% confidence limits (CLs). The median lethal time (LT$_{50}$) of each JHA was calculated using the ViStat 2.1 program.[20] Statistical differences in LT$_{50}$ among the JHA insecticides were determined by one-way analysis of variance (ANOVA) using SPSS 14 for Windows at $p < 0.01$. The competitive reaction of each JHA and JH III for the binding pocket of JHE from _S. littoralis_ and _S. frugiperda_ was statistically analyzed using SPSS 14 for Windows with least significant differences (LSD) at $p < 0.01$. The difference in JHE activity resulting from JHA topical application was also performed using LSD at $p < 0.01$ and $p < 0.001$.

### 3. Results

#### 3.1. Bioassays

The mortality response of 2nd instar _S. littoralis_ to continuous feeding exposure to methoprene, fenoxycarb, and pyriproxyfen is shown in Table 1. The LC$_{50}$ and LC$_{90}$ values indicated that fenoxycarb is more toxic to 2nd instar _S. littoralis_ than pyriproxyfen or methoprene. Methoprene showed the highest LC$_{50}$ and LC$_{90}$ values suggesting that it is the least toxic of the JHAs tested. In terms of time to kill, fenoxycarb showed a median lethal time that was about 10% and 20% faster than that of pyriproxyfen and methoprene, respectively.

Topical application of methoprene, fenoxycarb or pyriproxyfen (1 μg/2 day-old 6th instar larva) extended the length of the last stadium of _S. frugiperda_ by 5.0 ± 1.0 days beyond that seen in control larvae (Fig. 2A). No statistical difference in the duration of the pupal stage was observed in JHA insecticide-treated pupae (1 μg/3 day-old pupa). A statistically significant percentage of the JHA treated larvae and pupae failed to undergo normal development (Fig. 2B). The treated larvae and pupae commonly died as larval–pupal intermediates and as malformed adults, respectively (Fig. 2C). These effects were significantly higher in fenoxycarb and pyriproxyfen treated insects in comparison to methoprene treated insects. Of the pupae that originated from the fenoxycarb and pyriproxyfen treated larvae, more than 50% were larval–pupal intermediates or otherwise malformed, whereas only 23% of the pupae from methoprene treated larvae were larval–pupal intermediates (Fig. 2C). Similarly, of the adults that emerged from the fenoxycarb and pyriproxyfen treated pupae, more than 50% showed malformations, whereas only 14% of the adults from methoprene treated pupae showed malformations (Fig. 2C).

### 3.2. Inhibition of JHE activity and competitive interaction

JH hydrolytic activity in hemolymph that was collected from 6th instars of _S. littoralis_ or _S. frugiperda_ is shown in Figs. 3A and 3B, respectively. The mass range of _S. littoralis_ on days 1, 2, and 3 of the 6th instar was estimated to be 400–600 mg, 601–900 mg, and >900 mg, respectively. The mass range of _S. frugiperda_ on days 1, 2, and 3 of the 6th instar was estimated to be 400–500 mg, 501–600 mg, and >600 mg, respectively. The highest JHE activity in the last stadium of _S. littoralis_ (11.0 ± 0.3 nmol of JH III acid formed min$^{-1}$ ml$^{-1}$) and _S. frugiperda_ (23 nmol of JH III acid formed min$^{-1}$ ml$^{-1}$) was found in larvae that were 700–800 mg and 500–600 mg, respectively. From these results, hemolymph was collected from _S. littoralis_ and _S. frugiperda_ that were 700–800 mg and 500–600 mg, respectively, for further investigations.

The median inhibitory concentration (IC$_{50}$) of methoprene, fenoxycarb, and pyriproxyfen as well as the esterase inhibitors OTPF and DFP against JHE activity in the hemolymph of _S. littoralis_ and _S. frugiperda_ is shown in Table 2. Of the JHAs tested, fenoxycarb showed the highest inhibitory activity against JHE activity from both _S. littoralis_ and _S. frugiperda_. Of all of the compounds that were tested OTPF showed 1000-fold higher potency than any of the other compounds including DFP. The IC$_{50}$ of OTPF against JHE activity from _S. littoralis_ and _S. frugiperda_ was 6.7 and 1.6 nM, respectively. OTPF showed 4-times more potency against JHE activity from _S. frugiperda_ than that from _S. littoralis_. Similarly, DFP was about 6-times more potent against JHE activity from _S. littoralis_ than from _S. frugiperda_, however, the IC$_{50}$ of DFP was 30,000-fold higher than that of OTPF.

#### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>Lethal concentrations (μg ml$^{-1}$ diet)$^1$</th>
<th>LT$_{50}$ (h)$^2$</th>
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<tbody>
<tr>
<td></td>
<td>50% (95% CL)</td>
<td>90% (95% CL)</td>
</tr>
<tr>
<td>Methoprene</td>
<td>10.0 (2.7–19.8)</td>
<td>167.0 ± 7.0*</td>
</tr>
<tr>
<td>Fenoxycarb</td>
<td>5.3 (1.1–10.0)</td>
<td>139.8 ± 3.8*</td>
</tr>
<tr>
<td>Pyriproxyfen</td>
<td>6.8 (2.2–16.5)</td>
<td>154.4 ± 4.2*</td>
</tr>
</tbody>
</table>

$^1$ Mortality was recorded at 10 days after continuous exposure to five concentrations of insecticide contaminated diet. LC$_{50}$ and LC$_{90}$ values are shown with their 95% confidence limits (95% CL).

$^2$ Median lethal time (LT$_{50}$) was determined following exposure to an LC$_{50}$ concentration of each insecticide in triplicate. The data were analyzed by one-way ANOVA using SPSS Statistics version 14 for Windows. Values that are followed by different letters within the column are significantly different ($p < 0.05$).
The inhibition assays indicated that methoprene, fenoxycarb, and pyriproxyfen are poor inhibitors of JHE activity from *S. littoralis* and *S. frugiperda*. In order to determine if these JHAs are able to compete with JH III for the binding pocket of JHE, competition reactions between JH III and methoprene, fenoxycarb, or pyriproxyfen were performed (Fig. 4). Initially, the diluted hemolymph from *S. littoralis* or *S. frugiperda* was incubated with JH III and each of the JHAs at a molar ratio of 1:1 (JH III:JHA; Fig. 4). Under this condition, the JHAs showed no inhibitory

Fig. 2. Effect of JHA exposure during the larval and pupal stages of *Spodoptera frugiperda*. The duration of the larval or pupal stage (A), percentage of the population and adults emerged normally (B), and morphology (C) are shown. Sixth instar, day 2 larvae or 3 day-old pupae were topically treated with 1 μg of methoprene (1610 μmol), fenoxycarb (1659 μmol) or pyriproxyfen (1556 μmol) in 2 μl of acetone. Control larvae or pupae were treated with 2 μl of acetone. The asterisk (*) and number sign (#) indicate a statistical difference (p < 0.001) in comparison to control and methoprene treated insects, respectively. In C, the images were taken at 5 days post treatment of larvae (row I) or 7 days post treatment of pupae (row II). The columns marked 1, 2, 3, and 4 show representative control, methoprene, fenoxycarb, and pyriproxyfen treated insects, respectively.

The inhibition assays indicated that methoprene, fenoxycarb, and pyriproxyfen are poor inhibitors of JHE activity from *S. littoralis* and *S. frugiperda*. In order to determine if these JHAs are able to compete with JH III for the binding pocket of JHE, competition reactions between JH III and methoprene, fenoxycarb, or pyriproxyfen were performed (Fig. 4). Initially, the diluted hemolymph from *S. littoralis* or *S. frugiperda* was incubated with JH III and each of the JHAs at a molar ratio of 1:1 (JH III:JHA; Fig. 4). Under this condition, the JHAs showed no inhibitory

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**Table 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (mean ± SD) of JHE from</th>
<th>DS2</th>
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</thead>
<tbody>
<tr>
<td></td>
<td><em>S. littoralis</em></td>
<td><em>S. frugiperda</em></td>
</tr>
<tr>
<td>Methoprene (μM)</td>
<td>935 ± 100b</td>
<td>1820 ± 240b</td>
</tr>
<tr>
<td>Fenoxycarb (μM)</td>
<td>9 ± 1b</td>
<td>50 ± 7c</td>
</tr>
<tr>
<td>Pyriproxyfen (μM)</td>
<td>&gt;25000</td>
<td>&gt;25000</td>
</tr>
<tr>
<td>OTFP (μM)</td>
<td>6.7 ± 1.0d</td>
<td>1.6 ± 0.6d</td>
</tr>
<tr>
<td>DFP (μM)</td>
<td>211 ± 13b</td>
<td>1210 ± 120b</td>
</tr>
</tbody>
</table>

1 The IC50 values were calculated using SigmaPlot 11 software. The data were analyzed by LSD using the statistical analysis program of SPSS version 14 for Windows. Within the same column, IC50 values that are followed by a different lowercase letters are significantly different (p < 0.001). The IC50 values of pyriproxyfen could not be determined because the highest percentage inhibition was only 49 ± 9% and 26 ± 3% for JHE activity from *S. littoralis* and *S. frugiperda*, respectively.

2 The degree of susceptibility (DS) of JHE from *S. littoralis* and *S. frugiperda* to each compound was determined by dividing the highest IC50 value by the lowest IC50 value of JHE from *S. littoralis* and *S. frugiperda*.

3 OTFP: 3-octylthio-1,1,1-trifluoro-2-propanone; DFP: diisopropylfluorophosphate.
or enhancing effects on JH III hydrolysis. In subsequent experiments, when the diluted hemolymph was incubated with JH III and each of the JHAs at a molar ratio of 1:10 (JH III:JHA; Fig. 4), fenoxycarb inhibited JHE activity from both *S. littoralis* and *S. frugiperda* by about 52% \( (p < 0.001) \) and 31% \( (p < 0.01) \), respectively.

### 3.3. In vivo effect of JHAs on JHE activity

JHE activity in the hemolymph of 6th instar *S. littoralis* and *S. frugiperda* following topical application of methoprene, fenoxycarb or pyriproxyfen is shown in Fig. 5. At 24 h post JHA treatment (1 μg/ larva in 2 μl of acetone), JHE activity in pre-wandering *S. littoralis* was between 9 and 20 nmol of JH III acid formed min\(^{-1}\) ml\(^{-1}\), and in *S. frugiperda* it was between 13 and 20 nmol of JH III acid formed min\(^{-1}\) ml\(^{-1}\). These values were 5- to 11-fold higher (*S. littoralis*) and 14- to 22-fold higher (*S. frugiperda*) than that found in the hemolymph of control larvae that were only treated with acetone. JHE activity in the hemolymph of 6th instar *S. frugiperda* at 24 h post JHA treatment was similar or slightly lower than that found in pre-wandering larvae immediately prior to JHA treatment (Fig. 5B) suggesting that JHA application was able to prevent the dramatic drop in JHE activity that was found in control larvae. On the other hand, JHE activity in the hemolymph of JHA-treated larval *S. littoralis* (at 24 h post JHA treatment) was higher than that found in pre-wandering larvae immediately prior to JHA exposure, respectively. The experiments were repeated 3 times for each treatment or control. The data were analyzed by one-way ANOVA using SPSS Statistics version 14 for Windows. Bars within each panel that are labeled with different letters are significantly different \( (p < 0.01) \).

### 4. Discussion

The roles of JH, JH mimics, and JH metabolism in larval–pupal transformation of lepidopterous larvae have been investigated in several pest insect species including the tobacco hornworm *Manduca sexta* and cabbage looper *Trichoplusia ni* [21–26]. However, the role of JH metabolism in the developmental biology of *S. littoralis* and *S. frugiperda* which are
major insect pests of a variety of crops in multiple countries [27–31] has not been exhaustively studied. A clear understanding of the biological role of JH metabolism is particularly important because JH mimics are increasingly used to control a wide range of lepidopteran pests [21,32]. To this end we carried out studies to evaluate the effects of methoprene, fenoxycarb, and pyriproxyfen on the development of S. littoralis and S. frugiperda as well as hemolymph JHE activity from these insects.

Morphologically, the presence of JHAs during the last larval instar can result in the formation of larval–pupal or pupal–adult intermediates [33–34]. In our study, we found that larvae that were exposed to fenoxycarb (1 μg) or pyriproxyfen (1 μg) were completely inhibited from switching from normal larval development to normal pupal and died as larvae. Similar results are observed in other lepidopteran insects such as Spodoptera littoralis [35], Bombyx mori [36,37], and Omphisa fuscidentalis [38]. On the other hand, following methoprene (1 μg) exposure, a portion of the larvae appeared to normally switch from larval to pupal development. In terms of pupal–adult transformation, apparently normal adults emerged from 86%, 40%, and 48% of methoprene, fenoxycarb or pyriproxyfen treated pupae, respectively (Fig. 2B). In pupal Tenebrio molitor, ecdysone titer is significantly reduced following treatment with pyriproxyfen [39]. Pyriproxyfen may also inhibit normal pupal development in lepidopterans by modulating the effects of 20-hydroxyecdysone (20E). Our findings suggest that a reduction in ecdysone titer following JHA exposure may affect larval–pupal or pupal–adult metamorphosis in lepidopterans. The reduction in ecdysone titer could result from a block in the release of prothoracicotrophic ecdysone [40] which in turn would result in a block in the secretion of ecdysone. These effects are observed in Bemisia tabaci, Trialeurodes vaporariorum [41], Alphitobius diaperinus, and Ostrinia nubilalis [42] that are treated with JHAs.

The importance of JH metabolism in the regulation of normal larval development and metamorphosis has been demonstrated using slow, tight binding, transition state analog inhibitors that block JHE activity [43,44]. For instance in M. sexta, the inhibition of JHE with a trifluoroacetone inhibitor results in giant larvae [8], a phenotype that is also found following JHA insecticide application [15]. In lepidopteran hemolymph, a decrease in JH titers is positively correlated with an increase in JHE activity, and JHE activity is responsible for reducing JH titers by more than 95% in larval T. ni [46]. In this study we found that OTP is a powerful inhibitor of the JHE activity from both S. littoralis and S. frugiperda, while DFP is a poor inhibitor (Table 2).

None of the JHAs that were tested in this study were potent inhibitors of JH activity. Fenoxycarb showed some inhibition of JHE activity from S. littoralis and S. frugiperda, however, this activity was much lower (IC50 of 9 μM and 50 μM, respectively) than that of OTP (low nanomolar IC50). Our in vitro competition assays showed that fenoxycarb but not pyriproxyfen or methoprene could significantly (p < 0.001 and p < 0.01) reduce the hydrolytic JHE activity from S. littoralis and S. frugiperda, respectively, when it was present in the assay at a 10:1 M ratio (i.e., 5 μM fenoxycarb to 0.5 μM JH III), while no reduction in JHE activity was found when present at a 1:1 M ratio. When similar experiments were performed with a recombinant JHE from the mosquito Culex quinquefasciatus both fenoxycarb and methoprene showed no inhibitory activity [13]. Taken together, our findings suggest that JHAs have little or no effect in altering the normal biological function of JHE. The ability of JHAs to inhibit other JH metabolic enzymes such as JH epoxide hydrolase or JH biosynthetic enzymes such as JH acid methyl transferase, however, needs further investigation.

In our study, the topical application of JHA insecticides maintained hemolymph JHE activity or even appeared to induce hemolymph JHE activity. In the case of S. frugiperda, JHE activity post-JHA treatment was the same as that found at the time of the JHA treatment. In comparison, a dramatic drop (by 5-fold to more than 20-fold) in JHE activity was found in control insects. In both S. littoralis and S. frugiperda, hemolymph JHE activity following fenoxycarb treatment was lower than that with methoprene or pyriproxyfen (Fig. 5). A similar effect is found in diamondback moths treated with pyriproxyfen which results in a 50% increase in JH activity even following exposure at a concentration as low as 10−9 mol l−1 [47]. These findings indicate that topical application of fenoxycarb induces JHE activity from S. littoralis and S. frugiperda. The induction of JHE activity that was found in this study also suggests that the tested JHA insecticides do not function as direct inhibitors of JHE from S. littoralis and S. frugiperda.

JH analog insecticides function by blocking larval–pupal transformation as well as normal pupal development. In this study, JHA-induced effects were observed following the treatment of two spodopterans with methoprene, fenoxycarb or pyriproxyfen. Of the three JHAs insecticides that were tested, pyriproxyfen was the most toxic and showed the fastest speed of kill followed by pyriproxyfen and then methoprene. Our results are consistent to those found in previous studies [17,48] and suggest that the JHA insecticides tested are able to maintain the status quo in spodopteran insects.

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References
