Ecdysteroid receptor docking suggests that dibenzoylhydrazine-based insecticides are devoid of any deleterious effect on the parasitic wasp *Psyttalia concolor* (Hym. Braconidae)

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Ecdysteroid receptor docking suggests that dibenzoylhydrazine-based insecticides are devoid of any deleterious effect on the parasitic wasp *Psyttalia concolor* (Hym. Braconidae)

Paloma Bengochea, Olivier Christiaens, Fermín Amor, Elisa Viñuela, Pierre Rougé, Pilar Medina and Guy Smagghe

Abstract

**BACKGROUND:** The moulting accelerating compounds (MACs) or ecdysteroid agonists represent a selective group of insecticides acting upon binding to the ecdysteroid receptor (EcR) and leading to lethal premature moulting in larval stages and aborted reproduction in adults. *Psyttalia concolor* Szépl. is a useful parasitic wasp attacking important tephritid pests such as the medfly and olive fruit fly.

**RESULTS:** Contact and oral exposure in the laboratory of female parasitic wasps to the dibenzoylhydrazine-based methoxyfenozide, tebufenozide and RH-5849 did not provoke negative effects. No mortality and no reduction in beneficial capacity were observed. The ligand-binding domain (LBD) of the EcR of *P. concolor* was sequenced, and a homology protein model was constructed which confirmed a cavity structure with 12 α-helices, harbouring the natural insect moulting hormone 20-hydroxyecdysone. However, a steric clash occurred for the MAC insecticides owing to a restricted extent of the ligand-binding cavity of the PcLBD-EcR, while they did dock well in that of susceptible insects.

**CONCLUSIONS:** The insect toxicity assays demonstrated that MACs are selective for *P. concolor*. The modelling/docking experiments are indications that these insecticides do not bind with the LBD-EcR of *P. concolor* and support the theory that they show no biological effects in the parasitic wasp. These data may help in explaining the compatible use of MACs together with parasitic wasps in IPM programmes.

**Keywords:** *Psyttalia concolor*; parasitic wasp; ecdysteroid receptor; moulting accelerating compounds; modelling; docking; side effects

1 INTRODUCTION

*Psyttalia* (Opius) concolor Szépligeti (Hym. Braconidae), which belongs to the *P. concolor* species complex, parasitises larvae of Tephritidae. Its host records are available for about 24 species. Members of the complex have been extensively used in both classical and augmentative biological control programmes against tephritid pests.

Members of the family of Tephritidae are among the most economically important pests of edible fruits worldwide. Species such as the medfly *Ceratitis capitata* (Wiedemann), the Oriental fruit fly *Bactrocera dorsalis* (Hendel), the olive fly *Bactrocera oleae* (Rossi), the Mexican fruit fly *Anastrepha ludens* (Loew) and various melon flies in the genera *Dacus* and *Bactrocera* all cause millions of dollars annually in control and monitoring costs. Interest in utilising parasitic Hymenoptera for management and control of pest tephritids dates back to the early 1900s when initial efforts were made to locate natural enemies for medfly in western Australia and for olive fly in Italy. Subsequent success in Hawaii following introductions against medfly in 1913 initiated a long series of studies on parasitic wasps that attack tephritid pests.

Nowadays, *P. concolor* is still routinely used in the Mediterranean region for augmentative releases against *B. oleae*. Testing the side effects of pesticides on non-target arthropods is necessary for integrated pest management (IPM) programmes. *P. concolor* has been widely used in many insecticide experiments because it has been found to be the most sensitive natural enemy out of a list of 22 studied species, represented by different orders and families.

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The ecdysteroid agonists or moulting accelerating compounds (MACs), such as tebufenozide (RH-5992), methoxyfenozide (RH-2485) and RH-5849, act upon binding with the ecdysteroid receptor (EcR). They are chemically described as substituted dibenzoylhydrazines (DBHs) and mimic the natural function of the endogenous insect moulting hormone 20-hydroxyecdysone (20E), inducing premature lethal moulting in larval stages and aborting reproduction in adults, especially in Lepidoptera and Coleoptera.7,8 Furthermore, good activity against mosquito larvae of *Aedes aegypti* (L.), *Culex quinquefasciatus* (Say) and *Anopheles gambiae* (Giles) has also been demonstrated.9

The functional receptor complex of 20E is a heterodimer of the products of EcR and ultraspiracle (USP) genes. Both proteins belong to the superfamily of nuclear hormone receptors, which consists of ligand-dependent transcription factors that share two conserved domains: the DNA-binding domain (DBD) and the ligand-binding domain (LBD). They play a central role in controlling gene expression during the development of arthropods. Their general modular structure commonly has four domains, namely the A/B, C, D and E domains, although some receptors also contain an F domain at the carboxy terminal end of the protein. The DBD and the LBD are the most conserved across all taxa for both receptors.10–12 Crystal structures of the LBD provide important information on the recognition of the ligands and the mechanisms of activation of nuclear receptors.13 MACs bind to the LBD of the EcR in susceptible insects, inducing premature moulting and affecting reproduction.7,8

In this study, the side effects of the three MACs methoxyfenozide, tebufenozide and RH-5849 on *P. concolor* have been tested. A guidance document for regulatory testing and interpretation of studies with non-target arthropods has been used. The document is the result of the activities of the Joint Initiative set up in 1994 by the International Organisation for Biological and Integrated Control of Noxious Animals and Plants (IOBC), the Beneficial Arthropod Testing Group (BART) and the European and Mediterranean Plant Protection Organisation (EPPO) in collaboration with the Council of Europe.14 Both exposure to a treated inert surface (worst-case laboratory conditions) and ingestion tests have been performed in the present study. The LBD of the EcR of this natural enemy (Pc-EcR) has been cloned and sequenced. A three-dimensional (3D) model of the LBD of *P. concolor* EcR (PcEcR-LBD) has been constructed to evaluate whether it exhibits the typical canonical structure with 12 α-helices. Finally, ligand docking has been performed to support the theory that DBH-based insecticides are devoid of any deleterious effect on the parasitic wasp *P. concolor*.

## 2 MATERIALS AND METHODS

### 2.1 Insects

*P. concolor* female adults were obtained from a mass-rearing culture maintained in the Laboratory of Entomology (Polytechnic University of Madrid, Spain), as described before.15 Mass-rearing cultures are carried out in a climate chamber under controlled conditions (25 ± 2 °C, 75 ± 10% RH and 16:8 (L:D) photoperiod).

### 2.2 Insect bioassays

Bioassays were carried out under the same conditions as mass-rearing cultures. The following active ingredients were tested: methoxyfenozide (Runner®), 24 SC; Dow Agrosciences, Madrid, Spain), tebufenozide (Mimic 2F®), 24 SC; Dow Agrosciences, Madrid, Spain) and RH-5849 (technical product, >95%; Rohm and Haas, Spring House, PA). A systemic insecticide, dimethoate (Dimetoato 40 Progress®<sup>16</sup>, 40 EC; Cheminova Agro S.A., Madrid, Spain), was used as a commercial standard, and distilled water as a control. Solutions of product were prepared freshly in distilled water prior to the assays, based on their respective maximum field recommended concentrations (MRFCs) in accordance with the Spanish registration, with a delivery rate of 1000 L water ha⁻¹.<sup>17</sup> Acetone was used as a solvent for RH-5849.

Experiments consisted of five replicates per treatment. Ten unfed, mated females (<48 h old) per replicate were used. Distilled water was provided *ad libitum* in small glass vials (15 mm diameter, 22 mm height) covered with Parafilm<sup>®</sup> and a piece of Spontex<sup>®</sup> wiper leaking out of it. Diet (consisting of icing sugar: brewer’s yeast; 4:1) was supplied in small plastic stoppers (24 mm diameter, 6 mm height). Mortality was recorded daily as the percentage of dead wasps. After 3 days of exposure, parasitic behaviour and beneficial capacity were evaluated over 5 days. For this, five females per replicate (five replicates per treatment when there were enough surviving females) were transferred to a round plastic cage (12 cm diameter plastic cage with two 5.5 cm diameter ventilation holes on the top and bottom, covered with mesh). During the following 5 days, 30 fully grown medfly *C. capitata* larvae were offered to each replicate for parasitisation. Larvae of *C. capitata* had been previously collected from the diet and put into water to avoid pupation before offering them to female wasps. They were immobilised by sandwiching them between the mesh of the cage’s floor and a piece of Parafilm<sup>®</sup> placed on the floor of a glass pot and held together with a rubber band. After 1 h of exposure, *C. capitata* larvae were transferred to petri dishes to let them pupate. Beneficial capacity was measured as the percentage of attacked hosts (percentage of puparia without medfly emergence) and progeny size (percentage of parasitoids emerged from parasitised puparia). Data of the first parasitisation day were rejected because previous experiments had shown that females needed 1 day before getting used to parasitising in their new cages.

**Exposure via ingestion.** To evaluate the oral toxicity of the insecticides, they were offered to females via their drinking water. Females were placed into plastic cages similar to those used during parasitisation measurement but with a single hole on the top. Mortality was evaluated over 3 days, and beneficial capacity was measured as previously described. Insecticides were also offered to females during parasitisation.

**Residual contact on glass surfaces.** To evaluate the residual contact activity of the insecticides, glass plates (12 × 12 × 0.5 cm) were treated under a Potter precision spray tower (Burkard Manufacturing Co., Uxbridge, UK). As soon as the glass plates were dried, test units were built with a methacrylate frame. Test units consisted of a round methacrylate frame (10 cm diameter, 3 cm height) and the two square glass plates described above. The plastic frame had eight holes (1 cm diameter): seven covered by a mesh for ventilation and the eighth holding a small rubber tube with a hypodermic needle at the end. The needle was connected to a bigger rubber tube providing a continuous flow of air produced by an aquarium pump to assure forced ventilation. Adults were introduced into each test unit, which were then mounted with two crossed rubbers. Mortality was measured over 7 days, and beneficial capacity was evaluated as described above.

Data [means values ± standard errors (SE)] were subjected to one-way analysis of variance (ANOVA), and Fisher’s least significant difference (LSD) test was used to compare responses at field rate concentrations. All statistical analyses were performed using
Table 1. Toxicity of methoxyfenozide, tebufenozide and RH-5849 to *P. concolor* females

<table>
<thead>
<tr>
<th>Compounds</th>
<th>% Mortality 72 h</th>
<th>% Attacked hosts</th>
<th>% Progeny</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingestion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>– 0 ± 0</td>
<td>99 ± 1</td>
<td>54 ± 7</td>
</tr>
<tr>
<td>Methoxyfenozide</td>
<td>96 0 ± 0</td>
<td>98 ± 1</td>
<td>59 ± 7</td>
</tr>
<tr>
<td>Tebufenozide</td>
<td>180 0 ± 0</td>
<td>99 ± 0</td>
<td>53 ± 5</td>
</tr>
<tr>
<td>RH-5849</td>
<td>180 4 ± 4</td>
<td>99 ± 0</td>
<td>41 ± 8</td>
</tr>
<tr>
<td>F (df)</td>
<td>– 1.0 (3, 16)</td>
<td>0.68 (3, 12)</td>
<td>1.27 (3, 12)</td>
</tr>
<tr>
<td>P</td>
<td>– 0.418</td>
<td>0.583</td>
<td>0.329</td>
</tr>
<tr>
<td>Residual contact</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>– 0 ± 0</td>
<td>94 ± 2</td>
<td>58 ± 13</td>
</tr>
<tr>
<td>Methoxyfenozide</td>
<td>96 2 ± 0</td>
<td>97 ± 2</td>
<td>55 ± 9</td>
</tr>
<tr>
<td>Tebufenozide</td>
<td>180 0 ± 0</td>
<td>90 ± 8</td>
<td>59 ± 11</td>
</tr>
<tr>
<td>RH-5849</td>
<td>180 0 ± 0</td>
<td>86 ± 8</td>
<td>54 ± 8</td>
</tr>
<tr>
<td>F (df)</td>
<td>– 1.00 (3, 16)</td>
<td>0.42 (3, 12)</td>
<td>0.06 (3, 12)</td>
</tr>
<tr>
<td>P</td>
<td>– 0.418</td>
<td>0.745</td>
<td>0.980</td>
</tr>
</tbody>
</table>

*Concentration of compounds is given in mg Al L−1.*

The systemic insecticide dimethoate, as commercial standard, caused 100% mortality at 24 h after the treatment.

Table 2. Degenerate and specific primers used to complete PceCR-LBD coding sequence

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward primer</th>
<th>Reversed primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GAAGTVATAGTGYTNMNGATG</td>
<td>AGCTCCCAKATYTCWCKNARVA</td>
</tr>
<tr>
<td>2</td>
<td>GVCVAAHTGYCARGAGTG</td>
<td>ATTTGTTTATCGGGGACGTG</td>
</tr>
<tr>
<td>RACE</td>
<td>CGAGGCACCTAGAACATAGC</td>
<td>GGCACCACCGTCGACTGATC</td>
</tr>
<tr>
<td>Bridge</td>
<td>GAACGCGCTACCTGGAGGTA</td>
<td>AGTGCCCGCTGTATTTGAAA</td>
</tr>
<tr>
<td>Cloning</td>
<td>CTGCAGCAGCTGCTCTGTA</td>
<td>CCACCTGGCGCAATTACCTG</td>
</tr>
</tbody>
</table>

* Abridged universal amplification primer (AUAP).

Statgraphics<sup>®</sup> v.5.1.16 When needed, data were transformed to arc sin √(x/100) or log (x + 1), depending on whether the data were expressed as percentages or not, even though non-transformed data are shown in Table 1. If any of the premises of analysis of variance were violated after appropriate transformations, the non-parametric Kruskal–Wallis test was applied. Median values were considered significantly different if 95% confidence intervals of medians did not overlap.

### 2.3 PceCR-LBD sequence and phylogenetic analysis

Total RNA was extracted from *P. concolor* adults using TRI reagent (Sigma-Aldrich, Bornem, Belgium), based on a single-step liquid-phase separation method. The quality and quantity of the extracted RNA were examined by gel electrophoresis and spectrophotometry using a Nanodrop<sup>®</sup> ND-1000 (Thermo Fisher Scientific, Asse, Belgium). Subsequently, first-strand cDNA synthesis was performed using SuperScript<sup>®</sup> II reverse transcriptase (Invitrogen, Merelbeke, Belgium) with the oligo(dT)<sub>12–18</sub> primers according to the manufacturer’s protocol.

The complete PceCR-LBD coding sequence was then determined through a number of PCR reaction steps. Partial sequences of the LBD were obtained using degenerate and specific primers (Table 2) located in the coding sequence of the LBD and the DBD were obtained using degenerate and specific primers through a number of PCR reaction steps. Partial sequences of the DBD and ending in the 3′ UTR, was cloned and sequenced for confirmation.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Forward primer</th>
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</thead>
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<tr>
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<td>GGCACCACCGTCGACTGATC</td>
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<td>CTGCAGCAGCTGCTCTGTA</td>
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</tr>
</tbody>
</table>

* Abridged universal amplification primer (AUAP).

The same *P. concolor* cDNA as that used in the identification of EcR-LBD was used for the initial PCR reactions of the cloning. After purification, the PCR products were ligated into a pGEM-T vector (Promega, Madison, WI) according to the manufacturer’s instructions. Afterwards, plasmids were transformed in competent *Escherichia coli* XL-1 blue cells by heat shock and then plated out on an ampicillin-containing LB (lysogeny broth) agar plate. After 16 h of incubation, formed colonies were checked by colony PCR, and several of these positive colonies were then purified using Plasmid Mini Prep kit (Omega Bio-Tek) and sent for sequencing (AGOWA).

The ECR-LBD sequences of several arthropods and two human orthologues of EcR were retrieved by Blast searches against the Genbank database. The chosen sequences were then aligned by CLUSTALW2/CLUSTALX2, and the phylogenetic trees were made using MEGA4 software. Bootstrap analysis with 1000 replicates for each branch position was used to assess support for nodes in the tree.

### 2.4 Confirmation of expression of EcR in *P. concolor* ovaries

RNA from dissected ovaries from female adults of *P. concolor* was extracted, and subsequently cDNA was synthesised following the same procedure described before for total RNA. The expression of the EcR in the ovaries was investigated by PCR using the same specific primers designed for the cloning process.

### 2.5 Modelling of PceCR-LBD and docking studies

Homology modelling of the PceCR-LBD was performed with the YASARA structure program running on a 2.53 GHz Intel core duo Macintosh computer. Different models were built from the X-ray coordinates of the EcR of the lepidopteran *Heliothis virescens* in complex with a synthetic DAH-based ligand as BYI-06 830 (PDB combination with a gene-specific primer at helix 9–10 in order to amplify and sequence the beginning of the LBD (fragment 2). Then, in a third step, the 3′ end of the transcript was eventually also amplified by RACE-PCR using the 3′ RACE system for rapid amplification of cDNA ends (Invitrogen), using a specific primer in the helix together with the abridged universal amplification primer (AUAP) that is delivered with the kit. This fragment was subsequently sequenced. Finally, a small missing part between helices 10 and 12 was also amplified and sequenced, and afterwards (bridge fragment) the whole fragment, starting in the DBD and ending in the 3′ UTR, was cloned and sequenced for confirmation.

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code 3IXP), the RXR-USP receptor of the coleopteran Tribolium castaneum bound to ponasterone A (P1A) (PDB Code 2NXX),22 the Ecr-LBD of the hemipteran Bemisia tabaci complexed to P1A (PDB code 125X),23 the Ecr-USP of H. virescens in complex with 20E (PDB code 2R40)24 and the human RXRα (PDB code 3FC6) used as templates.26 Finally, a hybrid model was built up from the five previous models. PROCHECK was used to assess the geometric quality of the 3D model.27 In this respect, about 87% of the residues of Pcecr-LBD were correctly assigned on the best allowed regions of the Ramachandran plot, the remaining residues being located in the generously allowed regions of the plot (result not shown). Using ANOLEA to evaluate the models,28 only five residues of Pcecr-LBD over 246 exhibited an energy level over the threshold value. All of these residues are located in the loop regions connecting α-helices. Molecular cartoons were drawn with YASARA and PyMol (DeLano WL, http://pymol.sourceforge.net).

Docking of 20E, P1A, tebufenozide and methoxyfenozide to Pcecr-LBD was performed with the YASARA structure program. Clipping planes of Pcecr-LBD complexed to the steroids 20E and P1A and the four DAH-based ecysedone agonists tebufenozide, methoxyfenozide, halofenozide and RH-5849 were rendered with PyMol.

3 RESULTS

3.1 Side effects of insecticides

As shown in Table 1, the three tested MACs did not cause any mortality on P. concolor females. No effects were detected either when females were exposed to a treated surface or when they ingested the product (P > 0.05). When exposed to dimethoate, however, 100% of females died after 24 h in both experiments.

Furthermore, no sublethal effects of the three MACs on reproductive parameters, namely the percentage of attacked hosts and progeny size, were observed in either assay (P > 0.05) (see Table 1), and no change in behaviour of treated wasps was seen.

3.2 Pcecr-LBD sequence, phylogenetic tree and expression in the ovaries

The cDNA encoding the Pcecr-LBD fragment was cloned in order to obtain the sequence. Figure 1 shows a multiple alignment with the amino acid sequence of Pcecr-LBD, together with the Ecr-LBD from most other known hymopteran species, and several members from other insect orders. Pcecr-LBD exhibits some amino acid substitutions in positions where conservation is usually very high throughout the class of Insecta. These residues are marked in Fig. 1 with red dots: residues at positions 2 and 16 in helix 1, at position 26 in helix 2 and at positions 54 and 70 in helices 3, 72, 113 and 195. In some cases, amino acid substitutions are observed that are similar to those in hemipteran species but different from those in hymopteran species, as indicated with blue dots in Fig. 1 (positions 104, 124, 140, 223 and 227). In fact, sequence identity analysis showed that the LBD of P. concolor exhibits a stronger sequence identity to the insect order of Hemiptera and also to the Coleoptera than to the Hymenoptera (Table 3).

Phylogenetic trees of the Ecr-LBD, including various insect species from several orders such as Diptera, Lepidoptera, Hymenoptera, Hemiptera, Orthoptera and Coleoptera, together with some Crustacea and Chelicerata, group Pcecr-LBD together with the Hemiptera, close to Nezaraviridula, instead of the Hymenoptera clade (Fig. 2). Maximum parsimony trees also confirmed this result (data not shown).

4 DISCUSSION

4.1 Bioassays

Evidence collected to date indicates that the MAC insecticides such as methoxyfenozide, tebufenozide and RH-5849 have an excellent margin of safety to non-target organisms, including a wide range of non-target and beneficial insects, as well as mammals, birds and fishes.29–31 The latter is in agreement with the results obtained in this study for the parasitic wasp P. concolor. These products also proved to be harmless when their residual contact activity on an inert surface was tested on the nymphs or adults of the predators of Orius laevigatus (Fieber), Macrophlus caliginosus (Warner) and Amblyseius californicus (McGregor).32 No deleterious effects were detected when the hemipterans O. insidiosus (Say), Podisus maculiventris (Say) and P. sagitta (F.) were exposed to relatively high doses of RH-5849 and tebufenozide either.33–35 Among parasites, they also proved to be safe for the parasitic wasps Encarsia formosa (Gahan),32 Hypoaspis dixymed (Thunberg),36,37 Telenomus remus (Nixon),38 Trichogramma cacoeciae (Marchal),39 T. pretiosum (Riley) and Allorhogas pyralophagus (Marsh).40,41 However, some studies have reported deleterious effects to certain coccinellid and hymenopteron biocontrol agents of economic importance in citrus orchards.42

Hitherto, no sublethal effects on the beneficial capacity of the parasitic wasps of P. concolor, namely their reproductive parameters, have been reported. In agreement with the present results, no adverse effects were detected on reproduction (production of males) or on the development of the larvae in the treated nests when MACs were applied to bumblebees [Bombus terrestris (L.) (Hym. Apidae)].31 In contrast, MACs strongly
Figure 1. Sequence alignment of ecdysone receptor ligand-binding domains (LBDs), including PcEcR-LBD. The alignment includes ecdysone receptors from Diptera (Drosophila, Aedes, Ceratitis), Lepidoptera (Bombyx, Junonia, Bicyclus), Coleoptera (Tribolium, Tenebrio, Leptinotarsa), Hymenoptera (Apis, Polistes, Nasonia, Acromyrmex, Camponotus, Bombus, Solenopsis, Myridole, Ptytalia) and Hemiptera (Nezara, Bemisia, Nilaparvata). Red dots indicate amino acid substitutions in PcEcR-LBD sequence in positions where conservation is usually very high throughout the insect class. Blue dots indicate amino acid substitutions in PcEcR-LBD that are similar to those in hemipteran species and different from the hymenopteran. Yellow dots indicate the amino acids involved in the ligand binding in the EcR-LBD. Amino acid colours indicate similar structure. The figure has been prepared using CINEMA.
Table 3. Sequence identity between PcEcR-LBD and the EcR-LBD in other insect orders (%)

<table>
<thead>
<tr>
<th>Insecta</th>
<th>Diptera</th>
<th>Lepidoptera</th>
<th>Coleoptera</th>
<th>Hymenoptera</th>
<th>Hemiptera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>65.3 (64–67)</td>
<td>58.7 (57–60)</td>
<td>81.0 (79–82)</td>
<td>77.9 (74–81)</td>
<td>79.7 (77–84)</td>
</tr>
</tbody>
</table>

Data are given as average. Data in brackets refer to the range. Species used are Drosophila melanogaster, Aedes aegypti, Ceratitis capitata, Bombyx mori, Junonia coenia, Bicyclus anynana, Tribolium castaneum, Tenebrio molitor, Leptinotarsa decemlineata, Apis mellifera, Polistes dominulus, Nasonia vitripennis, Acromyrmex echinatior, Camponotus japonicus, Bombus terrestris, Solenopsis invicta, Pheidole megacephala, Bemisia tabaci, Nilaparvata lugens and Nezara viridula.

Figure 2. Phylogenetic tree of LBD-EcRs. This tree was constructed by the neighbour-joining method using the amino acid sequences of the LBDs of the selected sequences. Bootstrap values as percentage of 1000 replicates of > 50 are indicated on the tree.

Effect of dibenzoylhydrazine-based insecticides on P. concolor

affect reproduction in sensitive insect species such as Lepidoptera and Coleoptera, which in many cases result in sterile female adults and/or abnormal genitalia, which hinder the mating process or the capacity to produce fertile offspring.7,8,43 The prevention or cessation of the oviposition of some Coleoptera, Lepidoptera and Diptera was also observed, and similar effects were reported for Hemiptera.29,44 Dissection of lepidopteran and coleopteran females that had been treated with MACs and stopped oviposition showed that the formation of new ovarioles seemed to be inhibited, and they already showed signs of degeneration, resulting in very frail ovarioles. However, all the eggs that had been deposited by the treated females were equally viable.33,34,45 In contrast, no alterations on oocyte growth or on the ovulation process were detected in tebufenozide-treated lacewing predatory adults.46

4.2 Sequence and phylogenetic analysis

To date, different sequences for the EcR-LBD in Hymenoptera are already available. For a number of social insects belonging to the Formicidae, Vespidae and Apoidae families, the sequence is known, but these three families comprise only a small part of the large order of Hymenoptera. Apart from these social insects,
Figure 3. (A), (B), (C) Overall three-dimensional conformation of the *Tribolium castaneum* TcEcR-LBD domain (A), the *Heliothis virescens* HvEcR-LBD domain (B) and the modelled LBD domain of the EcR receptor from the Hymenoptera *Psytallia concolor* (PcEcR-EcR) (C), all in complex with ponasterone A (P1A) (blue stick). The twelve $\alpha$-helices distributed along the polypeptide chain are numbered H1 to H12. (D), (E), (F) Clip view (dashed yellow line) of the ligand-binding pocket of TcEcR-LBD (D), HvEcR-LBD (E) and PcEcR-LBD (F) harbouring P1A (pink stick). (G), (H), (I) Network of hydrogen bonds (dashed blue lines) anchoring ponasterone A (P1A) to the TcEcR-LBD (G), HvEcR-LBD (H) and PcEcR-LBD (I). Residues interacting with the ligand by hydrophobic interactions are coloured orange. Residues are labelled according to the sequence alignment presented in Fig. 1. (J), (K), (L) Clip view (dashed yellow line) of the ligand-binding pocket of TcEcR-LBD (J), HvEcR-LBD (K) and PcEcR-LBD (L) harbouring 20-hydroxyecdysone (20E). (M), (N), (O) Network of hydrogen bonds (dashed blue lines) anchoring 20-hydroxyecdysone (20E) to TcEcR-LBD (M), HvEcR-LBD (N) and PcEcR-LBD (O). Residues interacting with the ligand by hydrophobic interactions are coloured orange. Residues are labelled according to the sequence alignment presented in Fig. 1.
Figure 4. (A), (B), (C) Clip view (dashed yellow line) of the ligand-binding pocket of the Tribolium castaneum TcEcR-LBD domain (A), the Heliothis virescens HvEcR-LBD domain (B) and the modelled LBD domain of the EcR receptor from the Hymenoptera Psytallia concolor (PcEcR-EcR) (C) harbouring the DAH-based ecdysone agonist tebufenozide (TEBU) (blue stick). Note the steric conflicts († and ‡) of tebufenozide with the wall of the ligand-binding pocket of PcEcR-LBD. (D), (E), (F) Network of amino acid residues of TcEcR-LBD (D), HvEcR-LBD (E) and PcEcR-LBD (F) interacting with tebufenozide (TEBU) by hydrogen bond (dashed blue line) and hydrophobic interactions. Hydrophobic and aromatic residues are coloured orange. (G), (H), (I) Clip view (dashed yellow line) of the ligand-binding pocket of TcEcR-LBD (G), HvEcR-LBD (H) and PcEcR-LBD (I) harbouring methoxyfenozide (METHO) (blue stick). Note the steric conflicts († and ‡) of methoxyfenozide with the wall of the ligand-binding pocket of PcEcR-LBD. (J), (K), (L) Clip view (dashed yellow line) of the TcEcR-LBD (J), HvEcR-LBD (K) and PcEcR-LBD (L) harbouring halofenozide (HALO) (blue stick). Note the steric conflict (†) of halofenozide with the wall of the ligand-binding pocket. (M), (N), (O) Clip view (dashed yellow line) of the TcEcR-LBD (M), HvEcR-LBD (N) and PcEcR-LBD (O) harbouring the agonist RH-5849 (BH) (blue stick). Note the steric conflict (†) of RH-5849 with the wall of the ligand-binding pocket.
only one EcR sequence is known for another hymenopteran, namely the Pteromalidae parasitoid wasp *Nasonia vitripennis* (Ashmead), the genome of which has recently been sequenced.\(^{57}\) Sequence alignment analysis with PcEcR-LBD indicated a number of substitutions in regions of the LBD that are usually strongly conserved. It also indicated a higher sequence identity to the Hemiptera than to the Hymenoptera. Phylogenetic analysis confirmed this, showing that PcEcR-LBD grouped together with the Hemiptera rather than with the Hymenoptera. Indeed, the EcR-LBD of *P. concolor* exhibits higher sequence identity on the amino acid level to most Hemiptera orthologues such as *Nezara viridula* (L.), *Bemisia tabaci* (Gennadius) and *Nilaparvata lugens* (Stal.) (77–84%, average 79.7%) than to the other Hymenoptera orthologues (74–81%, average 77.9%). However, these differences could also be partly caused by the limited amount of data that are available for non-social Hymenoptera. It is clear from the sequence data that, in some conserved regions, social hymenopteran insects, especially ants, have the same amino acid substitutions, while these are not shared by the EcR of the two parasitic wasps (*N. vitripennis* and *P. concolor*) or other insect species. Other examples of nuclear receptors not following the normal phylogeny have also been described before. For instance, the Mecopterida EcR proteins failed to cluster together with the rest of the Holometabola group, in spite of this being considered a monophyletic group.\(^{10}\) A similar phenomenon was found in Hemiptera, where members of the Sternorrhyncha suborder did not group together with the Heteroptera or Auchenorrhyncha suborders to form a hemipteran clade. The phylogenetic distance of PcEcR-LBD from that of *Lepidoptera* can explain the negative correlation with the high affinity of MAC for Lepidoptera. This negative correlation was also found for RH-5849 in *N. viridula*,\(^{11}\) the EcR-LBD of which exhibits a high sequence identity to PcEcR-LBD.

### 4.3 Modelling of PcEcR-LBD and docking studies

Although sequence conservation for the LBD of NRs, including the EcR, is high, small amino acid substitutions in this domain can have a major impact on the 3D structure of the protein, and in particular on the size and shape of the ligand-binding pocket. The residues involved in the ligand binding are indicated in Fig. 1, and it can be said that most of them are conserved in the sequences from different species.\(^{13}\) PcEcR-LBD exhibits a high conservation among these ligand-binding-involved residues. However, two of them are completely different from the other EcR-LBD sequences, namely threonine54 (helix 3) and methionine221 (helix 11), which in *P. concolor* are substituted by alanine and isoleucine respectively. In Lepidoptera, which show a high sensitivity for tebufenozide and methoxyfenozide, the divergent residues lining the binding pocket are the methionine56 and the valines 98 and 109. In the case of other insect and non-insect arthropods that show no or low susceptibility for tebufenozide and methoxyfenozide, these residues are substituted by isoleucine, methionine and isoleucine respectively. Here, it is of interest that this is also the case for *P. concolor*. In particular, the presence of an isoleucine at position 56 in non-sensitive species generates steric contacts between the \(\gamma\)-methyl group of the isoleucine residue and the C5-methyl group of the A-ring of the tebufenozide or the C4-ethyl group of its B-ring, depending on the orientation of the tebufenozide.\(^{48}\) This can explain the lack of toxicity of the MACs against *P. concolor*. Similar results have been reported for bees.\(^{51}\) Indeed, the present data are strong indications that target-site differences in the moultling hormone receptor play an important role. The latter hypothesis is consistent with the concept that the structure and biochemical properties of EcR may differ among insect species. However, it needs also to be mentioned here that, next to the structure of the EcR-LBD pocket, other factors such as pharmacokinetics and metabolic detoxification additionally play an important role in determining the biological spectrum of the MAC insecticides.\(^{48}\) For instance, the penetration of tebufenozide in non-sensitive *Chrysoperla carnea* (Stephen) (Neur. Chrysopidae) female adults was relatively slow and low, while the absorption in sensitive Lepidoptera was much more rapid.\(^{46}\) The latter results demonstrated that the low penetration and absorption patterns of tebufenozide also help to explain its non-toxicity towards *C. carnea* larvae.

### 5 CONCLUSIONS

In conclusion, this paper reports on the effects of three MACs on the parasitic wasp *P. concolor*. The data show no biological activity of methoxyfenozide, tebufenozide and RH-5849 on this important natural enemy. Modelling of the PcEcR-LBD and docking experiments also suggest that DBH-based insecticides such as tebufenozide and methoxyfenozide are devoid of any deleterious effect on the wasp. Thus, these products could be applied safely in IPM programmes in which the parasitic wasp is present, although it is recommended to test MACs also on other species to prevent undesirable effects on the auxiliary fauna.

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