CRIAÇÃO, RESPOSTAS BIOLÓGICA E COMPORTAMENTAL DE *Tetrastichus howardi* (OLLIFF) (HYMENOPTERA: EULOPHIDAE) A INSETICIDAS

por

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(Sob Orientação do Professor Jorge Braz Torres – UFRPE)

RESUMO

Tetrastichus howardi (Olliff) (Hymenoptera: Eulophidae) parasita pupas das brocas-da-cana, e de outras pragas. Atualmente são utilizados parasitoides de ovos e larvas para o controle das brocas e *T. howardi* visa adicionar mortalidade para a fase de pupa, a qual apresenta alta sobrevivência. Sua liberação em campo requer criação em larga escala, podendo utilizar pupas de *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) como hospedeiro alternativo. O uso de hospedeiro alternativo pode afetar o desempenho do parasitoide devido ao possivel condicionamento ao hospedeiro natal. Adicionalmente, a eficácia de *T. howardi* no controle da praga alvo, pode ser afetada por outras táticas de manejo, como aplicação de inseticidas. Preferência, parasitismo de pupas da praga alvo, *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae) e desempenho do parasitoide, foram avaliados mediante testes de escolha entre os hospedeiros (natal e alvo) por até 30 gerações de criação de *T. howardi* em pupas de *T. molitor*; foi determinada a produção de ovos em função da idade das fêmeas do parasitoide provenientes de ambos os hospedeiros (natal e alvo), produção de ovos após oviposições e o parasitismo da broca. Também, foi determinada a resposta biológica e comportamental do parasitoide a resíduos dos inseticidas clorantraniliprole, bifentrina, espinetoram, etiprole, fipronil e tiametoxam. A criação consecutiva de *T. howardi* em pupas de *T. molitor* não afeta seu desempenho nem a preferência pela praga alvo. A maturação sexual e produção de ovos por fêmeas *T. howardi* foi alcançada entre 72 e 96h de idade, e não foi observado recuperação na produção de novos ovos até 24h após o parasitismo; depois do parasitismo da pupa no interior do colmo o parasitioide abandona o hospedeiro. O inseticida clorantraniliprole foi compatível com *T. howardi*, enquanto os demais motraram-se tóxicos para o parasitoide. Entretanto, pupas da broca disponibilizadas em mudas de cana contendo resíduos de etiprole e espinetoram foram parasitadas, com produção de descendentes, bem como o parasitoide mostrou habilidade de evadir do ambiente com plantas tratadas com esses inseticidas.

PALAVRAS-CHAVE: Controle biológico, criação massal, hospedeiro alternativo, seletividade, comportamento.

REARING, BIOLOGICAL AND BEHAVIORAL RESPONSES OF *Tetrastichus howardi* (OLLIFF) (HYMENOPTERA: EULOPHIDAE) TO INSECTICIDES

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ABSTRACT

Tetrastichus howardi (Olliff) (Hymenoptera: Eulophidae) parasitizes the pupae of sugarcane borers and other pests. Sugarcane stalk borers are currently controlled using parasitoids of eggs and larvae, while *T. howardi* will target the pupal stage, which has a high survival rate lodging inside sugarcane stalks. *Tetrastichus howardi* release in the field requires a large-scale rearing, with the option of using *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) as an alternative host. However, the use of an alternative host can affect the performance of the parasitoid due to probable conditioning by the natal host. Furthermore, the effeciency of *T. howardi* in controlling the target pest can be supplemented by other management strategies, such as insecticide use. Preference, parasitism of pupae of the target pest, *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae), and biological quality of the parasitoid were evaluated using host choice tests (natal and target) for up to 30 generations of rearing *T. howardi* using pupae of *T. molitor*; egg loading concerning the age of parasitoid females from both hosts (natal and target); egg loading after oviposition; and behavior after parasitism inside the sugarcane stalk were assessed. In addition, the biological and behavioral responses of the parasitoid to residues of the insecticides chlorantraniliprole, bifenthrin, spinetoram, ethiprole, fipronil, and thiamethoxam were determined. Consecutive rearing of *T. howardi* in *T. molitor* pupae does not affect performance or preference for the target pest. Sexual maturation and egg loading of females were achieved between 72 and 96 hours of age, and there was a restraining in new eggs production within 24 hours after parasitism; after parasitism of the pupa inside the stalk, the parasitoid abandoned the host. The insecticide chlorantraniliprole was compatible with *T. howardi*, whereas the others harmed the parasitoid. However, pupae of the borer offered to parasitism on sugarcane seedlings containing residues of ethiprole and spinetoram were parasitized resulting in offspring production with the parasitoid showing the ability to escape from the environment with plants treated with these insecticides.

KEY WORDS: Biological control, mass rearing, alternative host, seletivity, behavior.

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CAPÍTULO 1

INTRODUÇÃO

A cana-de-açúcar (ou simplemente cana), *Saccharum officinarum* (L.), tem a Índia como centro de origem e foi introduzida no Brasil, em 1522 (Bastos 2018). Desde então, a cana se tornou uma das principais culturas, apresentando um papel importante na economia do país. A cana é usada principalmente para a produção de açúcar e etanol, além do aproveitamento dos subprodutos e resíduos para co-geração de energia elétrica, alimentação animal e fertilizante (Silalertruksa & Gheewala 2019).

O Brasil é o maior produtor mundial de cana-de-açúcar seguido pela Índia, Tailândia, China e Paquistão (OECD/FAO 2023). O constante aumento na demanda por etanol, as grandes áreas cultiváveis e condições edafoclimáticas favoráveis para esta cultura, projetam o Brasil como um forte produtor, consumidor e exportador mundial (CONAB 2019). Estima-se que na safra 2023/24, a área destinada ao cultivo de cana-de-açúcar seja de 8,410 milhões de hectares, com uma produção de 637,1 milhões de toneladas de cana crua, sendo 38,77 milhões de toneladas de açúcar e 27,53 bilhões de litros de etanol. Dados da safra 2022/23 mostraram como a região Sudeste se destaca como a principal produtora do país, com mais de 380 milhões de toneladas de cana processada, representando mais de 60% da produção nacional, principalmente nos estados de São Paulo e Minas Gerais. A região Centro-Oeste está no segundo lugar com 131,5 milhões de toneladas e o Nordeste em terceiro com 56 milhões de toneladas. Pernambuco, um estado tradicional produtor de cana desde o período colonial, obteve uma produção de 14,6 milhões de toneladas (CONAB 2023).

Como a maioria das atividades agrícolas, principalmente em sistemas de cultivo de monoculturas, a presença de artrópodes pragas é uma realidade que reduz a produtividade. Entre as pragas com maior impacto na produtividade dos canaviais de Pernambuco, destacam-se a broca-gigante, *Telchin licus licus* (Drury), a cigarrinha-da-raiz, *Mahanarva fimbriolata*, a cigarrinha-da-folha, *Mahanarva posticata* (Stal.), e as brocas-do-colmo, *Diatraea* spp. (Mendonça 1996, Marques *et al.* 2008).

Brocas do Colmo da Cana-de-açúcar *Diatraea* spp.

A broca-do-colmo, *Diatraea saccharalis* (Fabr.), possui ocorrência em todo o país (Mendonça *et al.* 1996), enquanto a broca-amarela, *Diatraea impersonatella* (= *flavipenella*) (Walker), predomina no Nordeste, com 99% das infestações em relação à *D. saccharalis* (Freitas *et al.* 2007, Silva 2013).

O desenvolvimento de *Diatraea* spp. é holometábolo, passando pelas fases de ovo, larva, pupa e adulto (Botelho & Macedo 2002). Os adultos vivem em média nove dias, dos quais as fêmeas ovipositam durante os seis primeiros dias da fase adulta, com máxima produção de ovos ocorrendo nos primeiros e segundos dias de vida (Freitas *et al.* 2007). As fêmeas depositam, em média, 430 ovos, os quais são colocados nas folhas do ponteiro, geralmente na bainha. Os ovos, de formato elíptico e coloração amarelada, são depositados em massas na posição imbricada, em quantidades que variam de 2 a 40 ovos. Uma vez que os embriões estão desenvolvidos, os ovos adquirem coloração amarelo-escura, nos quais podem ser visualizadas as cápsulas cefálicas das larvas em desenvolvimento (Freitas *et al.* 2007). A fase embrionária pode variar dependendo da temperatura, mas com duração média de 9 dias (Botelho & Macedo 2002). Depois da eclosão e durante o primeiro e segundo instar, as larvas alimentam-se do parênquima foliar fazendo galerias na nervura central e, posteriormente, perfuram e penetram o colmo da cana, na região macia do palmito na planta adulta ou coleto da planta jovem. As larvas confeccionam galerias e completam seu desenvolvimento larval passando por sete instares com duração de aproximadamente 34 dias (Freitas *et al.* 2007). As pupas são formadas no interior dos colmos com a cápsula cefálica voltada para a abertura da galeria e tem duração de 9 a 12 dias até a emergência dos adultos (Mendonça 1996, Freitas *et al.* 2007).

As larvas causam injurias diretas ao perfurar a cana jovem, provocando morte da gema apical, sintoma conhecido como “coração morto”. Além disso, perfurações na cana adulta produzem brotações laterais, enraizamento aéreo, atrofiamento dos entrenós e tombamento, levando a uma diminuição da produção agrícola e industrial (Mendonça 1996). Ainda, as brocas do colmo causam injúrias indiretas, pois as galerias formadas pela larva permitem a ocorrência de fitopatógenos, por exemplo, fungos como *Colletotrichum falcatum* (Went) e *Fusarium moniloforme* (Sheldon) causadores da podridão vermelha, doença que se caracteriza por inverter a sacarose presente nos colmos. Esta associação acarreta menor recuperação da sacarose pela indústria, diminui a pureza do caldo e o rendimento do álcool e açúcar (Botelho & Macedo 2002, Gallo *et al.* 2002, Pinto *et al.* 2006).

Controle Biológico das Brocas do Colmo da Cana-de-acúcar

No manejo integrado das brocas da cana-de-açúcar, o controle biológico aplicado tem sido amplamente utilizado mediante a criação de parasitoides em laboratório, com posterior liberação inundativa em campo (Pinto *et al.* 2006). O controle na fase de ovo é realizado através de liberações de *Trichogramma galloi* Zucchi. (Hymenoptera: Trichogrammatidae) e na fase de larva pela liberação de *Cotesia flavipes* (Cam.) (Hymenoptera: Braconidae) (Silva *et al.* 2012, Valente *et al.* 2016, Salamanca *et al.* 2023). Estudos mostram que o parasitismo de *C. flavipes* causa até 80% da mortalidade em larvas e o parasitismo de *T. galloi* alcança mais de 89,4% em ovos de *D.*

impersonatella, em condições controladas (Pinto 2016, Valente *et al.* 2016). A associação desses dois parasitoides pode reduzir em mais de 60% a população de *D. saccharalis* (Botelho *et al.* 1999). Além da utilização dos parasitoides de ovos e larvas, o parasitoide *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae) tem sido encontrado parasitando pupas de *Diatraea* spp. (Cruz *et al.* 2011, Vargas *et al.* 2011), tornando-se uma espécie com registro para comercialização contra esta praga (AGROFIT 2023). Em laboratório, o parasitismo de *T. howardi* em pupas de *Diatraea* spp. é superior a 90%, com emergência média de 53 descendentes por pupa parasitada, com um período de desenvolvimento de aproximadamente 22 dias e razão sexual de 91% de fêmeas na descendência (Rodrigues *et al.* 2021).

Tetrastichus howardi Como Agente de Controle das Brocas do Colmo da Cana-de-Açúcar

O parasitoide *T. howardi* é originário da Ásia e encontra-se amplamente distribuído no mundo (La Salle & Polaszek 2007). Foi introduzido na África do Sul para o controle das brocas *Chilo partellus* (Swinhoe) (Lepidoptera: Pyralidae) e *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae), pragas de grande importância econômica no milho e no sorgo (Skoroszewski & Van Hamburg 1987, Kfir 2001, Rao *et al.* 2001). Também, foi introduzido nos Estados Unidos e em Cuba, com a finalidade de controlar *Diatraea* spp.em diferentes culturas (Bennett 1965, Álvarez *et al.* 2008). No Brasil, este parasitoide foi encontrado em campo, parasitando pupas de *Plutella xylostella* (L.) (Silva-Torres *et al.* 2010) e pupas de *Diatraea* spp. (Cruz *et al.* 2011, Vargas *et al.* 2011).

Pertencente à subfamília Tetrastichinae, *T. howardi* é um endoparasitoide cosmopolita e que possui desenvolvimento gregário. Tem como característica o hiperparasitismo facultativo, sendo seu desenvolvimento registrado mais frequentemente em diferentes espécies de Lepidoptera (Silva-Torres *et al.* 2010, Vargas *et al.* 2011, Pereira *et al.* 2015, Barbosa *et al.* 2015, Piñeyro *et* *al.* 2016). Os adultos apresentam coloração escura, com brilho metálico (La salle & Polaszek 2007). Nesta espécie, o dimorfismo sexual é evidenciado principalmente pelas antenas que, nas fêmeas apresentam o funículo pigmentado, com três segmentos, além de escapo sem placa sensorial na margem ventral. Nos machos, o funículo apresenta quatro segmentos e só a clava é pigmentada. Além disso, o escapo apresenta uma placa sensorial na margem ventral (La salle & Polaszek 2007, González 2004).

O comprimento do corpo varia de 1,6 a 2,2 mm nas fêmeas e 1,3 a 1,8 nos machos. O ovipositor é do tipo himenopteriforme, de coloração branco-leitosa, com 0,28 a 0,30 mm de comprimento e 0,08 mm de largura. Os ovos são depositados no interior do hospedeiro e demoram cerca de dois dias até a eclosão. As larvas apresentam três instares e o período larval tem duração aproximada de seis dias até a pupação. A fase de pupa dura entre 8 a 9 dias e, de cada pupa de *Diatrea* spp., emergem em média 53 indivíduos de *T. howardi*. A longevidade dos adultos varia de 15 a 25 dias. A duração de ovo a adulto dura entre 16 a 18 dias. O tamanho e o número de larvas, pupas e adultos dependem da espécie e tamanho do hospedeiro, assim como do número de larvas por hospedeiro (González *et al.* 2003, Costa *et al.* 2014ab, Rodrigues 2021).

O parasitoide *T. howardi* possui características importantes para ser considerado como um agente no programa de controle biológico das brocas-da-cana: (i) pode parasitar larvas, mas tem preferência por pupas (Vargas *et al.* 2011, Pereira *et al.* 2015, Rodrigues *et al.* 2019), adicionando mortalidade a uma fase da praga não alcançada pelos demais parasitoides comercializados; (ii) tem a capacidade de localizar e parasitar pupas em campo (Kfir *et al.* 1993, Barbosa *et al.* 2019); (iii) é capaz de se desenvolver e parasitar em temperaturas entre 18 e 32 °C (Favero *et al.* 2015) compatível com regiões produtoras de cana no Brasil; (iv) pode ser criado em laboratório utilizando diversos hospedeiros alternativos como pupas de *Erinnyis ello* (L.) (Lepidoptera: Sphingidae) (Barbosa *et al.* 2015), *Bombyx mori* (L.) (Lepidoptera: Bombycidae) (Piñeyro *et al.*

2016), *Anticarsia gemmatalis* Hübner (Lepidoptera: Erebidae) (Fernandes 2018) e *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) (Vargas *et al.* 2011, Oliveira 2013, Barbosa *et al.* 2019, Tiago *et al.* 2019), entre outros.

Uso de *Tenebrio molitor* como Hospedeiro Alternativo de *Tetrastichus howardi*

Larvas e pupas de *T. molitor* são usadas amplamente como presa e hospedeiro para a criação de inimigos naturais (Oliveira 2013, Glaeser *et al.* 2014, Zanuncio *et al.* 2008, Moreira *et al.* 2016, Morais 2016, Hu *et al.* 2017), por apresentarem baixo custo e fácil manutenção em criações de larga escala. Dessa forma pupas de *T. molitor* também são viáveis para a criação do parasitoide *T. howardi* (Vargas *et al.* 2011, Oliveira *et al.* 2014, Barbosa *et al.* 2019, Tiago *et al.* 2019). Estudos anteriores demonstram que este parasitoide apresenta melhor desenvolvimento em pupas de *T. molitor* expostas ao parasitismo com até 24h de idade e na densidade parasatoide-pupa de 7:1, sob condições controladas de temperatura de 25 ºC, umidade relativa de 70% ± 10, e fotofase de 14h (Oliveira 2013).

Condicionamento Pré-imaginal: Influência do Hospedeiro Natal no Parasitismo da Praga Alvo

Para localizar o hospedeiro e realizar a oviposição, os parasitoides utilizam pistas (Vinson 1976, Godfray 1994), como voláteis e outras características do hospedeiro ou da planta atacada (Poelman *et al.* 2012, Turlings & Wäckers 2004, Heil 2008, Becker *et al.* 2015). Ainda, para se desenvolver internamente, o parasitoide necessita neutralizar a defesa humoral do hospedeiro, criando uma associação que pode ser alterada com o efeito maternal. Estudos mostram que parasitoides podem ser condicionados ao hospedeiro de criação (Corbet 1985, Silva-Torres *et al.* 2005, Siqueira *et al.* 2012). Assim, existe uma dúvida frequente sobre o comportamento do parasitismo, após sucessivas gerações de criação em um hospedeiro alternativo (Liu *et al.* 2017) e seu potencial de controle após liberação em pragas alvo.

Devido ao aprendizado e memória que o indivíduo adquire em qualquer fase de seleção do hospedeiro (Dauphin *et al.* 2009), incluindo a aprendizagem pré-imaginal, logo na emergência dos adultos (Cortesero & Monge 1994, Gandolfi *et al.* 2003), os parasitoides tendem a usar pistas do hospedeiro natal na busca por novos hospedeiros (Van Emden *et al.* 1996, Davis & Stamps 2004, Verschut 2017). O condicionamento pré-imaginal é considerado um evento prévio à habituação da plasticidade, o que leva finalmente a uma evolução nas espécies (Bologna & Di Giulio 2011, Ramírez *et al.* 2016), consequentemente, populações de parasitoides que são criados durante consecutivas gerações em um único hospedeiro alternativo poderiam mostrar variação nas suas características biológicas, assim como na preferência pelo hospedeiro.

Uso de Inseticidas na Cultura da Cana-de-açúcar e os Efeitos Sobre o Parasitoide

Embora o uso de inseticidas no controle das brocas da cana-de-açúcar seja uma tática questionável pela baixa probabilidade de controlar suas populações, devido ao desenvolvimento parcialmente protegido dentro do colmo (Gitahy *et al.* 2006), pragas encontradas na cultura como cigarrinhas, formigas, cupins e o bicudo-da-cana de acúçar são alvos de aplicações com diferentes inseticidas (diamidas, espinosinas, fenilpirazois, pirazois e neonicotinoides) (AGROFIT 2023).

Inseticidas aplicados no canavial podem interagir de várias formas com os parasitoides quando liberados em campo. Um efeito direto sobre os parasitoides é a morte ou a sobrevivência e, quando o parasitoide sobrevive, indirectamente os resíduos dos inseticidas podem interferir no comportamento de busca e parasitismo. Alguns dos efeitos subletais que podem sofrir os parasitoides expostos a inseticidas incluem: mudança no comportamento, limitação na habilidade de forrageamento e capacidade de orientação, diminuição da longevidade e redução da taxa oviposição (De Jiu & Waage 1990, Stapel *et al.* 2000, Desneux *et al.* 2004, Desneux *et al.* 2007). Portanto, o uso de inseticidas sintéticos para o controle de pragas deve seguir critérios e a escolha do inseticida deve se basear na sua eficácia no controle da praga alvo e na segurança dos organismos não alvo, incluindo os inimigos naturais (Oliveira *et al.* 2014, Pedigo 1999).

Apesar de *T. howardi* se apresentar como um agente de controle biológico, para ser multiplicado em larga escala em diferentes hospedeiros e utilizado no controle biológico de *Diatraea* spp., é necesario conhecer o desempenho deste parasitoide, após sucessivas gerações de criação empregando pupas de *T. molitor* como hospedeiro alternativo. Além disso, é preciso identificar os efeitos dos inseticidas recomendados para pulverização em cultivos de cana-de-açúcar sobre *T. howardi*, sendo esta informação relevante para o sucesso na integração dos métodos de controle químico e biológico do manejo integrado das brocas-da-cana.

Portanto, os objetivos deste trabalho foram avaliar a preferência entre os hospedeiros (natal e alvo) e o desempenho no parasitismo de *T. howardi* sobre *D. saccharalis,* após ser criado durante 30 gerações em pupas de *T. molitor*; conhecer sobre a produção de ovos em função da idade da fêmea do parasitoide, a capacidade de produção de novos ovos das fêmeas após o parasitismo, assim como o comportamento depois de parasitar seu hospedeiro dentro do colmo da cana. Além de determinar os efeitos dos resíduos de inseticidas recomendados para o controle de pragas da cana-de-açúcar sobre o parasitoide.

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CAPÍTULO 2

REARING *Tetrastichus howardi* (OLLIFF) (HYMENOPTERA: EULOPHIDAE) TO ASSESS AN ALTERNATIVE HOST[1]

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[1] Bermúdez, N.C. Rearing *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae) to assess an alternative host. To be submitted.

ABSTRACT - The continuous use of an alternative host, sometimes taxonomically different from the target pest, can affect the performance of the parasitoid over successive generations due to conditioning in the natal host. *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae) can be reared using the pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) as an alternative host. Parasitoids were reared using *T. molitor* pupae for 30 consecutive generations, and quality criteria were evaluated during the generations F5, F15, and F30, offering pupae of the target pest, *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae), and compared with the F0 generation (parasitoids reared in *D. saccharalis* pupae). The experiments included assessments of parasitism performance and host selection (natal and target pest). Additionally, females *T. howardi* emerged from both hosts were examined for egg loading in relation to their age and egg loading after ovipositions, as well as parasitism of sugarcane stalk borer pupae inside the stalks. Rearing *T. howardi* using pupae of *T. molitor* did not affect its biological quality or its preference for the target pest after rearing consecutively for 30 generations. Female sexual maturation and egg loading occurred between 72 and 96 h of age, and egg loading recover after parasitism did not occur within 24 h period. The parasitoid leaves the host pupa inside the stalk after parasitism, and one oviposition was enough to kill *D. saccharalis* pupae obtaining a viable parasitism. Finally, *T. howardi* can be consecutively reared for up to 30 generations using the alternative host without decreasing parasitism performance and exhibit similar egg loading emerging from the alternative host.

KEY WORDS: Applied biological control, quality control of natural enemies, sugarcane borer

CRIAÇÃO DE *Tetrastichus howardi* (OLLIFF) (HYMENOPTERA: EULOPHIDAE) PARA AVALIAR UM HOSPEDEIRO ALTERNATIVO

RESUMO **-** O uso de hospedeiro alternativo, às vezes taxonomicamente diferente da praga alvo, pode afetar o desempenho do parasitoide devido ao condicionamento no hospedeiro natal. *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae), pode ser criado usando pupas de *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) como hospedeiro alternativo. Sendo assim, parasitoides foram criados por 30 gerações consecutivas em pupas de *T. molitor* e avaliados critérios de qualidade nas gerações F5, F15 e F30, ofertando pupas da praga alvo, *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae) e comparados com a geração F0 (parasitoides criados em pupas *D. saccharalis*). Os experimentos incluíram teste de escolha entre ambos hospedeiros (natal e praga alvo) e o desempenho de parasitismo. Adicionalmente, foi avaliada a produção de ovos em relação à idade das fêmeas de *T. howardi*, criadas em ambos os hospedeiros, e produção de ovos após oviposições realizadas, assim como o comportamento de parasitismo de pupas da praga no colmo da cana. A criação de *T. howardi* em pupas de *T. molitor* não afetou sua qualidade biológica nem sua preferência pela praga alvo, a maturação sexual e carregamento de ovos das fêmeas é atingida entre 72 e 96h de idade, não sendo observado recuperação da produção de ovos em um período de até 24h após o parasitismo. A fêmea do parasitoide abandona a pupa dentro do colmo após o parasitismo e uma oviposição é suficiente para matar a pupa de *D. saccharalis* e obter produção de descendentes. Ainda, *T. howardi* pode ser criado continuamente por até 30 gerações usando o hospedeiro alternative sem perda do desempenho de parasitismo e similar maturação sexual e produção de ovos quando criado no hospedeiro alternativo.

PALAVRAS-CHAVE: Controle biológico aplicado, controle de qualidade de inimigos naturais, broca-da-cana

Introduction

Applied Biological Control (ABC) relies on natural enemies reared on a large scale with quality and feasible costs. The development of artificial diets and methodologies using artificial or natural diets for rearing the target pest as a host is the first milestone for ABC (Leppla 1984). This step had many contributions, including reduction of contamination in the insectary, constancy of host/prey availability, feasible costs, and others (Leppla & Ashley 1989). Artificial diets used to rear the hosts and adaptations of rearing methodologies have progressed and contributed to ABC with parasitoids against different agricultural and veterinary pests. A second and parallel step was the use of high-quality and low-cost alternative hosts for rearing parasitoids, eliminating the dependency on continuous rearing of the target pests in the insectaries.

ABC has been strengthened by the use of alternative hosts with practicable costs with different species of *Trichogramma* worldwide, including *Sitotroga cerealella* Olivier (Lepidoptera: Gelechiidae), *Ephestia kuehniella* Zeller (Lepidoptera: Pyralidae)*, and Ostrinia furnacalis* Guenée (Lepidoptera: Crambidae) (Pratissoli *et al*. 2010, Moghaddassi *et al.* 2019, Li *et al.* 2019). Pupae of the yellow mealworm *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) have been a host for rearing *Sclerodermus guani* Xiao & Wu (Hymenoptera: Bethylidae) to control the Japanese pine sawyer, *Monochamns altevnatus* Hope (Coleoptera: Cerambycidae) (Hu *et al.* 2017). Lately, yellow mealworm pupa have been adopted to rear the parasitoids *Trichospilus diatraeae* Cherin and Margabandhu, *Palmistichus elaesi* Delvare and LaSalle, and *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae) (Pereira *et al.* 2021), registered to release against lepidopteran defoliators of *Eucaluptus* and sugarcane stalk borers (AGROFIT 2023).

Tetrastichus howardi is a cosmopolitan and gregarious endoparasitoid that naturally parasitizes different lepidopteran pest species (Silva-Torres *et al.* 2010, Vargas *et al.* 2011, Pereira *et al.* 2015, Barbosa *et al.* 2015, Piñeyro *et al.* 2016). Life history and behavioral features put forward *T. howardi* as a parasitoid candidate to recommend against pupae of sugarcane stalk borers. Parasitism occurs on larvae but preferentially on pupae (Vargas *et al.* 2011, Pereira *et al.* 2015), a stage of the sugarcane borers not targeted by egg and larval parasitoids in use; the female parasitoid finds the sugarcane borer pupa inside the sugarcane stalk in the field (Kfir *et al.* 1993, Barbosa *et al.* 2019); fulfills development and accomplishes parasitism when reared at temperature regimes between 18 and 34 °C (Yan *et al.* 2021); and can be reared in the laboratory on pupae of different species as alternative hosts (Barbosa *et al.* 2015, Piñeyro *et al.* 2016, Fernandes 2018), including pupae of T. molitor (Vargas *et al.* 2011, Oliveira 2013, Barbosa *et al.* 2019, Tiago *et al.* 2019).

To produce 1000 pupae of *T. molitor* reared on starter poultry feed, it was estimated to cost 28.57 US dollars, with an average of 130.5 parasitoids of *T. howardi* produced per pupa, resulting in an estimated cost of 0.00022 US dollars per adult parasitoid (Machado *et al.* 2023). Although *T. howardi* can be reared using pupae of various species of Lepidoptera (Pereira *et al*. 2021), *T. molitor* pupae have offered assets for commercial rearing of *T. howardi* (Tiago *et al.* 2019, Machado *et al.* 2023). This species is less demanding in terms of diet and rearing conditions, can be easier to rear in large quantities, and is also commercially available if needed to supply host scarcity for large-scale rearing. However, while pupae of Lepidoptera species provide a close relationship with the target pest, the sugarcane borer, *T. molitor,* is a Coleoptera and thus could affect the parasitoid's performance toward the target pest.

Gregarious eulophids can remain with the conciliated host after their location and hence perform multiple ovipositions (Matthews *et al.* 2009, Silva-Torres *et al.* 2010, Pereira *et al.* 2017). Female *T. howardi* parasitizes larger hosts such as sugarcane borer and yellow mealworm pupae (Rodrigues *et al.* 2021). Furthermore, the host finding relies on clues emitted by the host location (Vinson 1976, Godfray 1994). In addition, eulophids deal with host humoral defense in order to parasitize successfully (Andrade *et al.* 2010, Tang *et al.* 2019). Thus, these external cues and internal defense relationships can be altered by the parental host (Silva-Torres *et al.* 2005). Therefore, using alternative hosts for a long rearing period might affect parasitoid host finding and parasitism performance (Liu *et al.* 2017). Learning and memory that the individual acquires at any stage of host selection include pre-imaginal learning soon after the emergence of adults (Cortesero & Monge 1994, Gandolfi *et al.* 2003, Dauphin *et al.* 2009). If negative effects take place regarding host finding, acceptance, and parasitism performance, there will be consequences for ABC using alternative hosts (Van Emden *et al.* 1996, Davis & Stamps 2004, Verschut *et al.* 2017) for rearing *T. howardi* for long generations.

The preference and performance of the parasitoid *T. howardi* on pupae of *D. saccharalis* after continuous rearing on pupae of the alternative host, *T. molitor*; the egg loading according to the age of females from pupae of *D. saccharalis* compared to the alternative host, *T. molitor*; the number of ovipositions required to carry out a viable parasitism; and the capacity to produce new eggs after parasitism were determined. In addition, it was evidenced by the behavior of the female after parasitizing pupa of sugarcane borer inside the stalk. Therefore, four hypotheses were tested: Rearing *T. howardi* up to 30 generations using the alternative host, pupae of *T. molitor*, would not affect its choice of the target host or parasitism performance (i); parasitism of sugarcane borer, a larger host-pupa, will require *T. howardi* females successful ovipositision, reducing the egg loading, hence restrain further host parasitism (ii); female *T. howardi* would require a resting period after ovipositions to restablish egg loading (iii); females of *T. howardi* after finding a sugarcane borer pupa inside the stalk would restrain its dispersal from this pupa (iv).

Material and Methods

Insect Population Sources and Rearing Methods. A sugarcane borer colony, *D. saccharalis*, was established in the "Laboratório de Controle Biológico de Insetos" of the "Universidade Federal

Rural de Pernambuco" (UFRPE), Recife, Pernambuco State, Brazil, using pupae donated by the Associação de Plantadores de Cana da Paraíba (ASPLAN), located in the district of Pitanga da Estrada, Mamanguape, PB, Brazil. The physical conditions were 22 ± 1 °C, 70 ± 10% R.H., and a photoperiod of 12:12 h L:D.

Larvae of *D. saccharalis* were reared using an artificial diet prepared after Hensley & Hommond (1968). The diet consisted of soybean meal, wheat germ, sugar, vitamin solution, Wesson salts, ascorbic acid, water, and anti-contaminants (Nipagin and antibiotics). The rearing procedures were according to Fonseca *et al.* (2015). Briefly, newly hatched larvae were transferred to vials (7.5 × 14 cm in diam × Ht) containing an artificial diet. After 30 days of rearing, larvae were transferred to plastic boxes (30 × 18 × 4 cm in L × W × Ht), where they received diet until pupation. Pupae were collected and transferred to acrylic boxes (8.5 × 8.0 cm) lined with filter paper containing a wet cotton pad inside bottle caps until adult emergence. The adults were fed a 10% honey-water solution, provided in moistened cotton inside bottle caps put on the bottom of the rearing cages, made of cylindrical tubes of PVC measuring 22 × 20 cm (Ht × diam) and lined with paper for oviposition. In order to clean the eggs, they were submerged for three minutes in a solution containing 1% copper sulfate and 3% formalin, followed by a rinse in distilled water. After 5–6 days of incubation, eggs were put in Petri plates (2 15 cm in Ht diam) where larvae were employed to start a new rearing cycle.

The yellow mealworm, *T. molitor*, has been maintained in the same laboratory since 2016. Larvae and adults are reared on a diet consisting of 97% wheat flour and 3% yeast, as described in Torres *et al.* (2006). Vegetables such as slices of carrots, sweet potatoes, or open slices of sugarcane stalks are offered as part of the diet. Pupae served for parasitoid rearing and colony maintenance.

The parasitoid *T. howardi* colony was started with parasitized *D. saccharalis* pupae donated by the Laboratório de Controle Biológico de Insetos (LECOBIOL) at the Universidade Federal da Grande Dourados (UFGD), Dourados, MS, Brazil. After emergence, adult parasitoids were transferred to glass tubes (10 × 2 cm in Ht × diam) at a rate of seven females per vial, offered honey droplets as food, and closed with plastic film. The rearing procedure for *T. howardi* followed Vargas *et al.* (2011) using *T. molitor* pupae (≤ 48 h old). Each vial with parasitoid females received one *T. molitor* pupa for three days. After that, the parental females were discarded, and the pupae were reared until adult parasitoid emergence.

Sugarcane Plants. Sugarcane variety RB04-1443 was cultivated using cement rings (1.0 × 0.6 m in Diam × H) filled with soil inside a greenhouse under local natural physical conditions of 12–13 h photophase, 23–30 oC, and watered when as required. Plants were collected when they were around 10 months old, and the median region of the stalks, which had a diameter of 3.5 to 4.0 cm, was used. The collected stalks were cleaned, transported to the lab, and made ready for the sugarcane borer larvae and *T. howardi*'s parasitism.

Could the Use of Alternative Host Impair the Parasitism Performance of *Tetrastichus howardi*? The objective was to assess the parasitoid performance while being continuously reared on *T. molitor* pupae. To strengthen the naïve state of the parasitoid regarding the alternative host, *T. howardi* was first reared for five generations using pupae of the target pest, *D. saccharalis*. The descendents were then considered the F0 generation for the alternative host and used for comparisons with parasitoids reared using the alternative host. The F0 generation parasitoids were continuously reared using pupae of the alternate host, *T. molitor*. Its performance was evaluated at generations F5, F15, and F30 of rearing using *T. molitor*. Females of *T. howardi* were submitted to free-choice host selection and offspring production at F0, F5, F15, and F30 generations of rearing using the alternative host, pupae of *T. molitor* and offered *D. saccharalis* (TD standing for parasitoids emerged from *T. molitor* offered *D. saccharalis*), except for the experiment with parasitoid females at F0 generations, which were reared with pupae of *D. saccharalis* and exposed to pupa of *D. saccharalis* (DD). The treatments were set up using pupae of either the alternative host or the target host in Petri dishes (12 × 2 cm in Diam × H) lined with filter paper. Therefore, a test was conducted in which the parasitoid female was given free option over pupae from both hosts at the same time. The free-choice experiment had 20 replications, with hosts pupae 24 h old and of approximate size (weight) to minimize skewing resource availability. Two hours after the photophase began at 8 a.m., 48-hour-old, honey-fed, and mated *T. howardi* females were released into the arena. The females were continually monitored in order to record the host handling time, the time until the first choice, and the first choice for parasitism. The unselected pupa was carefully removed after the female parasitoid had chosen the host (the introduction of the ovipositor), and the female parasitoid was retained until the host pupa's death. The selected pupa was reared in order to measure the size of the offspring female, sex ratio, number of offspring produced, and parasitism rate.

Female wing and leg morphometry were measured by taking a random sample of three females per parasitized *D. saccharalis* pupa with adult emergence. These females were killed by freezing and used for taking the measurements. The right forewing and right hindleg were photographed using a digital camera that was connected to a stereomicroscope, a DIGILAB DI-150B (5X magnification). When needed, the quality of each image was enhanced using the software GIMP V.2.10.30. Images of the right forewings and hindlegs of the same 42, 24, 36, and 36 females that emerged at F0, F5, F15, and F30 generations, respectively, were captured. Wing geometric morphometry was obtained, defining 18 landmarks (LM) (Fig. 1S) placed on the digitalized images with TPSUtil v.1.74 and TPSdig2 v.2.30 (Rohlf 2017). To run the morphometric analysis, all traits were digitalized twice in order to test every outline against definition error through the performance of a Procrustes ANOVA, comparing the values of the mean squares of the error component of variation and individuals. All wings were subjected to a generalized Procrustes superposition using the program MorphoJ v.1.07a (Klingenberg 2011) in order to exclude the influence of size, position, and orientation in obtaining the shape variables (Rohlf & Slice 1990). With these variables, a covariance matrix of the individual shapes was performed to proceed with all corresponding multivariate analyses.

A Principal Component Analysis (PCA) was carried out individually for each of the digitalized trait covariance matrices in order to replicate and visually represent the shape space (Pearson 1901). Following the identification of related groupings, the classifier group was used to conduct a discriminant analysis using the Canonical Variate Analysis (CVA). Mahalanobis and Procrustes distances were used to calculate a permutation test (10,000 runs) between groups and display the corresponding *P*-values. Multivariate regression analysis was used with procrustes coordinates as the dependent variable and the size centroid as the independent variable to investigate the impact of size on the wing shape (allometry) (Monteiro 1999).

For traditional morphometry, the size of the wings and legs was determined using the Software TPSdig2 v.2.30 (Rohlf 2017). The wing length was considered from landmark one to landmark 15, and the wing width was considered from 13 to 18 landmarks (Fig. 1S). To assess the size of the tibia was considered the length between the junction of the tibia with the femur and the joint of the tarsus.

Egg Loading in *Tetrastichus howardi* as a Function of Age, Natal Host, and Ovipositions.

Aging and natal host (bioassay i)

The pre-oviposition period and reproductive performance are determined by the female parasitoid egg load. In this experiment, the target host, *D. saccharalis,* and an alternative host for rearing, *T. molitor*, were used as parental hosts. Females of *T. howardi* were dissected to determine the quantity of eggs generated in the ovaries as a function of age and parental hosts. Honey droplets put on the inside walls of the vial were given to *T. howardi* adults that emerged from *D. saccharalis* or *T. molitor* pupae as food, and mating was permitted up until the time of collection. Females representing the six female ages of 0-24, 24-48, 72-96, 96-120, and 120-144 hours were collected and analyzed. The succeeding aging dates were established from the collection of all females that fit the criteria for that age before 10 a.m. (i.e., 0-24 h old, honey-fed, and mated). These age intervals are 24, 48, 72, 96, 120, and 144 hours old for data characterization. At each stage of aging, females were randomly aspirated into 1.5-mL microcentrifuge tubes, killed by freezing at -10 ^{o}C, and kept there until dissection. The abdomen of the dead female was separated from the body using a razor blade, the contents of the abdomen were then extracted from the integument using two needles, and a coverslip was placed over the female abdome and applied light pressure. All of this was done under the light steremicroscope MOTIC SMZ-168 (5X-magnification). The number of eggs was calculated taking into account those that had a distinct form.

Sequential Ovipositions – same host pupa (bioassay ii)

In the field crop, likely one female of *T. howardi* will find the host pupa and might require multiple subsequent ovipositions to kill a larger host, hence experiencing an egg shortage for further parasitism. A parasitoid may also continue superparasitizing and injecting venom into the host after the first oviposition. This will increase the progeny's chance of survival by preventing the encapsulation and melanization of the eggs and overcoming the host's immune system (Pennacchio & Strand 2006; Andrade *et al.* 2010). In order to determine the number of females' viable ovipositions (Exp. 2.1) and the recovery of egg loading following parasitism (Exp. 2.2), two experiments were carried out.

Exp. 2.1 Females of *T. howardi*, 48 hours old, mated and honey-fed, emerged from one of the two parental hosts, *D. saccharalis* or *T. molitor*, were evaluated. The number of ovipositions was assessed using pupae of *D. saccharalis* 24–48 hours old. Pupae on the of molt were separated in Petri dishes (3.5 × 1 cm in Diam × H) to be used 24 hours late. The next day, one female parasitoid 48 hours old was released with a single pupa and, after that, continuously observed during 10 hours of photophase (8 a.m. to 6 p.m.). Females were assigned to accomplish up to one, two, or three ovipositions on the same pupa (treatments). An oviposition event consisted of observing the female mounting, the insertion of the ovipositor into the host, extraction, and finally moving away from the host (Rodrigues *et al.* 2021). Each treatment (number of ovipositions from one to three) was determined with 10 females (replications). Females, after accomplishing the assigned number of ovipositions on the same pupa, were collected, stored in a 1.5-mL microcentrifuge tube, and stored at -10 ^{o}C until dissection. In the control group, naive females 48 hours old, honey-fed, and mated were killed at -10 ^{o}C and had the number of eggs in the ovary counted. As previously described, females were dissected to count how many eggs were remaining in the ovary.

To verify the parasitism and the quantity of offspring generated, the pupae exposed to the parasitism were reared. In order to calculate the rate of parasitism, pupae without adult moth or parasitoid emergence were dissected. Additionally, parasitism also considered dead pupae with mummified parasitoids inside as a function of the number of ovipositions by a single female to infer about the unviable parasitism due to the host nourishment and defense.

Exp. 2.2 Female parasitoids emerging from both *D. saccharalis* and *T. molitor* natal hosts were subjected to observation of parasitism on *D. saccharalis* pupae during 10 hours of photophase in order to confirm the restoration of egg loading. Females who successfully completed three ovipositions were then taken away from their hosts and given 24 hours to rest without parasitism. These females were killed at -10 ^{o}C after the 24-hour resting period and dissected to count the number of eggs in the ovary (Fig. 2S). Again, honey-fed and mated 48-hour-old naïve females served as the control group for the dissection process.

Sequential ovipositions – different host pupa (bioassay iii)

In order to understand *T. howardi* performance when given the chance of successive ovipositions, 96-hour-old mated and honey-fed females were allowed two sequential ovipositions within a 10-hour photophase of continuous observations. Females were one by one aspirated from rearing vials and single-released into Petri dishes (3.5 × 1 cm in Diam × H) containing one pupa of *D. saccharalis* 24–48 hours old. Right after the parasitism, each pupa was removed from the dish, and another *D. saccharalis* pupa was added to allow a second oviposition. The observations were run for 10 hours in photophase with 31 parasitoid females. After that, each pupa with a parasitism attempt was reared separately until the adult emergence of either sugarcane borer or parasitoid. Pupae without the emergence of either one were dissected to certify the cause of the mortality.

Does Female of *Tetrastichus howardi* Stay with the Sugarcane Borer Pupa? In order to understand the parasitism of the sugarcane borer pupae inside the sugarcane stalk by *T. howardi*, this experiment exposed pupae housed inside sugarcane stalk pieces to parasitism. Pieces of stalks 18–20 cm in length with nodes at both ends were cut from the wider media portion of the stalks. A gallery 3 cm deep was made in the stalk section between nodes using a power drill with a 5mm diameter drill. After that, the piece of stalk was carefully opened longitudinally into two parts to facilitate placing the larvae inside the gallery at the deepest point of the gallery without damaging the larvae or during the assessments. The two parts of the stalk were tigheted together with the aid of rubber bands to avoid any opening other than the hole made using the drill, faking the entrance of the sugarcane borer into the stalk. The larvae were 30 days old and, therefore, stayed feeding for about 5 days before molting to pupa, which allowed building the natural conditions inside the gallery, such as feeding residues and feces. The pieces of stalk housing the larvae were positioned vertically with the node facing the opposite direction from the entrance hole inside an 80-mL plastic pot that contained moistened cotton to delay decay, and each piece of stalk housing one served as one observation unit. Each unit was enclosed by 3L plastic bottle cages with two lateral openings fixed with organdie fabric for ventilation. Once the larvae reached the pupal stage, six units of stalks, each housing a single pupa, were transferred to and placed vertically on a platform made with Styrofoam® board 50 × 32 cm (L × W) fixed inside a searching cage made with transparent Plexiglass® 50 × 45 × 32 cm (L × H × W). The finding and parasitism of the pupae inside the units were assessed with 10 replications (ca. 10 cages and 60 units). Each cage held six units and received the release of 84 parasitoid females to attain a rate of 14 females per available pupa.

To minimize stress on the female parasitoid, the assessments were conducted at night (in the dark) with the use of red light. These evaluations were made at 3, 6, and 9 days intervals after releasing the female parasitoid inside the cages. The number of parasitoid females on the pupa or inside the gallery was counted at each evaluation interval, and the stalks were marked to indicate whether the female was present or absent. Stalks with the presence of the parasitoid female with the pupa were immediately closed and individually returned to the observation unit, as previously described, and monitored daily until the ninth day to record the behavior of the female staying or abandoning the gallery. On the 9th day of exposure, the stalks observed in the units with previous records of female presence with the pupa were carefully opened, and the number of parasitoid females inside the stalks was recorded. The pupae were transferred to glass vials and reared until either adult moth or parasitoid emergence. Furthermore, the number of emerging parasitoids per pupa and the sex ratio of the offspring were recorded.

Statistical Analysis. All results of the choice test experiment were analyzed through deviance analysis (ANODEV) performed by Generalized Linear Models (GLM). The distribution utilized in the analyses was selected according to the nature of the data and the dispersion adjustment of the models. Thus, results referring to first choice, percentage of pupa parasitized, and effectively parasitized pupae were submitted to a GLM analysis using a binomial error distribution (link=logit). The time to first choice, the proportion of sex ratio, and morphometric variables were analyzed using a GLM with a Gaussian error distribution (link=identity). When necessary to observe the difference between generations of *T. howardi* that emerged in the same natal host, the data were submitted to a contrast analysis, making a comparison of means between the levels of this variable (F0, F5, F15, and F30) ($\alpha = 0.05$). Finally, the variables of time spent on the pupa and the number of offspring were analyzed using a GLM with a Poisson distribution (link=log), when it was necessary to adjust the model with a quasi-poisson distribution of the error. A residual analysis was performed on each model to ensure that the error distribution and model building were adequate. The analyses were performed using the software R (R Core Team 2020).

The number of eggs present in the ovary of naive females when reared with *D. saccharalis* or *T. molitor* was regressed against the age of the females (24, 48, 72, 96, 120, and 144 h) using PROC REG of SAS (SAS Institute 2002). Furthermore, to test the hypothesis that females might have their egg load changed due to the natal host, the linear portion of fitted models was compared. Thus, the linear slopes of fitted models (i.e., first- or second-order models) of the number of eggs per female age were compared between parental hosts using PROC MIXED to test the equality of linear slopes (SAS Institute 2002).

The number of eggs remaining in the ovary of *T. howardi* females emerged from either natal hosts after after 24 hours from zero, one, two, or three sequential ovipositions on *D. saccharalis* pupa; the number of offspring produced per parasitized pupa; and the offspring production per parasitized pupa from these three sequential ovipositions were transformed into square root (x+0.5) to meet the analysis of variance (ANOVA) assumptions and submitted to a two-way ANOVA with natal host and number of ovipositions as factors (SAS Institute 2002). Furthermore, the percentage of parasitism and mortality due to parasitism, the percentage of dead pupa due to parasitism with the emergence of parasitoids, and the number of parasitoids produced per parasitized pupa were tested for the hypothesis of equal performance between the 1st and 2nd successive ovipositions by a single *T. howardi* female using a chi-square test at 0.05 significance levels.

Results

Could the Use of Alternative Host Impair the Parasitism Performance of *Tetrastichus howardi*? The performance of *T. howardi* in finding and accepting the target pest, pupae of *D. saccharalis*, was unaffected by up to 30 generations of rearing it in the alternate host, *T. molitor* pupae. Females emerging from *T. molitor* pupae prefer the *D. saccharalis* pupae over the *T. molitor* pupae alternative host ($\chi^2 = 94.33$, $P < 0.0001$). Additionally, during all assessed generations of rearing the parasitoid in the alternate host, the choice for the target pest was maintained (Fig. 1). With the exception of generation F0, which used *D. saccharalis* pupae as both the natal host and the target host, the time needed for the first choice for parasitism was comparable throughout generations of rearing using the alternative host. Compared to females from later generations, F0 generation females made that first decision faster (Table 1). The other annotated variables, such as handling time, parasitism rate, the number of offspring produced, and the sex ratio, did not show any significant change across the generations of rearing using the alternative host (Table 1).

The morphometric outpu determined that using the alternative host to rear *T. howardi* over several generations had no impact on the size of the female's wing ($F_{3,\ 134}$ = 1.14, P = 0.32, Fig. 2A) by comparing the maximum length to the maximum width of the forewing. Females' hindleg tibias, on the other hand, varied in length through generations ($F_{3,\ 134}$ = 7.10, $P < 0.0001$, Fig. 2B), with larger tibias for females from the F5 and F30 generations.

The Procrustes ANOVA demonstrated that the mean square (MS) and *F*-values of error (MS = 0.000012, F = 0.69) were lower for the group than obtained for individuals (MS = 0.00016, F = 13.09). This shows that the landmarks were correctly digitized. According to the PCA estimated for the wing shape, the first three components (PC1 = 36.3%, PC2 = 14.2%, and PC3 = 9.1%) represented around 59.6% of the overall variance in wing shape. The PC1 indicates the average of wing shape variation regarding host switch between *Diatraea-Diatraea* (DD) and *Tenebrio-Diatraea* (TD) (Fig. 3A) and across generations (Fig. 3B), while the PC2 more clearly distinguishes the groups (both for host and for generation) (Fig. 3AB). It is easy to see that the shape of the wing of the F0 generation (DD) changed more noticeably between generations, with the change being identified in the distal part of the wing (Fig. 2S). The wing morphometry for generations F5, F15, and F30 shows a very little variance, but it still closely resembles the typical wing shape connected to PC1 (Fig. 2S). According to the CVA, two sets of wing morphometry were produced, one of which was composed of female wings from the F5, F15, and F30 generations, while the other was composed of female wings from the F0 generation (DD) (Fig. 4). Significant variations in wing shape were discovered using Mahalanobis and Procrustes distances (Table 1S). A link between wing size and shape was not shown by multivariate regression analysis (P = 0.63), with just 0.48% of the diversity in wing shape being explained by wing size.

Egg Loading in *Tetrastichus howardi* as a Function of Age, Natal Host, and Ovipositions.

Aging and natal host (bioassay i)

Regardless of the parental host, the egg load increased linearly as function of female ages when emerged from either host *D. saccharalis* pupae or *T. molitor* pupae (Fig. 5). The pattern of egg loading by naïve *T. howardi* females was consistent with female age (PROC MIXED of SAS for equality of linear coefficient, b1-b2 = -0.027, df =1, 115, t = -0.43, $P = 0.668$) (Fig. 5). For females emerging from *D. saccharalis* and *T. molitor* pupae, the mean (SE) number of eggs found in the ovary at mature age was 107.0 ± 2.9 and 99.5 ± 3.5 eggs, respectively.

Sequential Ovipositions – same host pupa (bioassay ii)

Exp. 2.1 The number of offspring produced per parasitized pupa of *D. saccharalis* per one *T. howardi* female was similar between the natal hosts ($F_{1,\,54} = 0.53$, $P = 0.47$), and the three sequential ovipositions ($F_{2,\,54} = 0.26$, $P = 0.76$), with a lack of interaction between these factors ($F_{2,\,54} = 0.65$, $P = 0.52$) (Fig. 6A). On the other hand, the remaining eggs in the ovary of females differed between natal hosts ($F_{1,\,72} = 6.61$, $P = 0.012$). When two ovipositions were done, females emerging from pupae of *D. saccharalis* kept more eggs in their ovary than females emerging from pupae of *T. molitor* (Fig. 6B). Furthermore, as expected, females performing one to three ovipositions had a significantly lower number of eggs present in the ovary compared to females without oviposition ($F_{3,\,72} = 143.87$, $P < 0.0001$). Despite that, the outcome shows no interaction with the parental host and sequential ovipositions for eggs remaining in the ovary of females ($F_{3,\,72} = 0.62$, $P = 0.061$).

Exp. 2.2 Females originated from either natal hosts and, after completing two or three ovipositions, had a similar number of eggs remaining in the ovary. Furthermore, the number of offspring produced per parasitized pupa of *D. saccharalis* by these females completing one, two, or three successful ovipositions by the same female on the same pupa was similar between natal hosts ($F_{1,\,54} = 0.04$, $P = 0.81$), the sequence of ovipositions ($F_{2,\,54} = 0.31$, $P = 0.73$), and the lack of interaction between these factors ($F_{2,\,54}$ = 2.23, P = 0.12) (Fig. 6C). On the other hand, females completing one, two, or three ovipositions and allowing a 24-hour recovery interval without parasitism showed variations in the eggs counted in the ovary regarding the natal hosts ($F_{1,\,54}$ = 4.63, P = 0.03) and the sequence of ovipositions ($F_{2,\,54}$ = 5.44, P = 0.007), but not for the interaction of these factors ($F_{2,\,54}$ = 1.82, P = 0.17) (Fig. 6D). Females that emerged from the alternative host, *T. molitor* pupa, had more eggs in the ovary after completing one oviposition and allowed a 24-hour recovery period (Fig. 6D).

Sequential ovipositions – different host pupa (bioassay iii)

Female *T. howardi* significantly reduced parasitism of pupae after accomplishing a 1st oviposition, with only 48.4% of the females accomplishing a 2nd oviposition within the 10-hour photophase period (Table 2). Females accomplishing a 2nd parasitism attempt successfully killed almost all pupae (93.3%). Despite that, only 35.7% of these killed pupae in the 2nd oviposition produced offspring. Furthermore, there was about a 50% reduction in offspring production from parasitized pupa in the 2nd oviposition.

Does Female *Tetrastichus howardi* stay with the Sugarcane Borer Pupa? Two pupae migrated out of the 60 larvae that were put within sugarcane stalks throughout the metamorphosis, leaving 58 pupae inside the stalk subject to parasitism. During a nocturnal check of these pupae, five pupae (ca. 8.6%) were found with *T. howardi* females inside the sugarcane stalk. However, within a day, these females abandon the pupa. Thirty-three of the 58 pupae (ca. 56.9%) died inside the stalk, with viable parasitism accounting for 45.3% of those pupae and 11.6% of pupae without parasitoid emergence. An average of 137.1 ± 12.8 parasitoids were generated per parasitized pupa, with a sex ratio of 0.89 (ca. 89% females), and the overall parasitism of pupae with descendent production was, on average, 45.3 ± 10.4% (mean ± SE).

Discussion

Enhancing the biological control of different target pest species with *T. howardi* will require large-scale rearing, which can be accomplished with an alternative host. However, the use of an alternative host for extended periods may result in conditioning to that host (Antolin *et al.* 2006, Henry *et al.* 2008, Li *et al.* 2010), which may impair parasitism performance (Jones *et al.* 2015, Samková *et al.* 2021). In spite of these assumptions, the parasitoid *T. howardi* did not change its choice of target pest, *D. saccharalis*, or make it less effective at parasitizing it. Furthermore, one oviposition of the parasitoid on the pupa of *D. saccharalis* was enough to achieve success in parasitism. One oviposition by female considerably reduces the egg load, and the female cannot recover the egg load within a period of 24 hours of rest. However, the lack of eggs in the ovary appear to have little effect on the female dispersal from the parasitized pupa.

Parasitoids use chemical and physical cues to locate and accept a host; consequently, the natal host might influence parasitoid performance on the target host (Godfray 1994, Poelman *et al.* 2012, Becker *et al.* 2015). Across all experiments carried out using the alternative host, *T. molitor*, the parastoid, *T. howardi*, preferred the pupae of *D. saccharlis* and achieved comparable or superior results parasitizing its target host. Similar results were found by Woltering *et al.* (2019). These authors found no decrease in the performance of the parasitoid *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae) reared for several generations on *Drosophila melanogaster* Meig. (Diptera: Drosophilidae), as an alternative host, when offered the target *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), in the laboratory. As a result, the findings suggest that rearing *T. howardi* in an alternative host (Coleoptera) for up to 30 generations, despite being taxonomically distant from the field target hosts (Lepidoptera), will not affect its biological performance and sounds feasible as an alternative host for mass rearing of *T. howardi*.

Another standard parasitoid quality criterion is body size. Larger size in parasitoids is generally linked to greater longevity and fecundity (West *et al.* 1996), with size influenced by host size (Silva-Torres *et al.* 2009), a common result with parasitoid eulophids (West *et al.* 1996, Silva-Torres & Mattews 2003, Liu *et al.* 2007). In this study, the length of the tibia was greater for F5 and F30 generations of rearing in the alternative host compared to females in the F0 generation that emerged from the target host. Similarly, Vargas (2013) found variation in the size of the cephalic capsule of *T. howardi* among 25 generations of continuous rearing using the pupae of *D. saccharalis*, with this variation observed between generations.

In the same sense, although the size of the wings did not differ between the females of the different generations reared in *T. molitor*, these showed a natural variation in shape, but with similar size and without deformations. Ray *et al.* (2016) showed that modification of *Drosophila* wing shape by genetic manipulation, which was much greater than natural variation, did not affect flight ability. Furthermore, Kölliker-Ott *et al.* (2004) assessed dispersal and host finding by *Trichogramma brassicae* Bezdenko (Hymenoptera: Trichogrammatidae) after large-scale rearing over a long period and found that wing size mattered but not wing shape. Thus, it is suggested that the differences in size and shape found in our study are a natural variation not influenced by the rearing host and that, possibly, did not have harmful consequences for the parasitoid when released in the field.

Eulophid parasitoids are often synovigenic, and unlike pro-ovigenic species, they need feeding in the adult stage to achieve egg maturation (Jervis *et al.* 2001, Zhang *et al.* 2011). All *T. howardi* females reared and used in the tests were honey-fed, therefore this requirement was met. Female *T. howardi* had around 50% of the egg load within 24 hours of adulthood but increased egg production with age. This indicates a gain in ovogenesis through time, a characteristic of synovigenic parasitoids. Knowing about this parasitoid reproductive strategy will help select which method to apply to improve parasitoid performance in the field, such as utilizing mated and fed to release females.

Females of *T. howardi* 72 hours old reared using *D. saccharalis* or *T. molitor*, had an average of 72 and 93 eggs formed in the ovary. These results are consistent with those from Zhong *et al.* (2016), who found females of *T. howardi* with first eggs 12 hours after emergence, but mature eggs were only observed after 24 hours and at 72 hours. These authors also found that females produced on average 71.6 mature eggs. However, females in our study continued producing eggs, reaching maximum production at 120 and 144 hours when reared with *T. molitor* and *D. saccharalis*, respectively. Therefore, females of *T. howardi* require between 72 and 96 hours to complete sexual maturity and egg loading, which is an intrinsic trait and independent of the natal host. This knowledge makes it possible to improve the large-scale breeding of this parasitoid using females at an age with maximum reproductive potential.

Irrespective of the number of ovipositions on the same pupa, a similar number of offspring was produced per parasitized pupa of *D. saccharalis*. In fact, a single oviposition of *T. howardi* killed the sugarcane borer pupa and produced a standard number of descendants, refuting the hypothesis that multiple ovipositions would be required to guarantee success in parasitism of this pest. This finding follows Sequeira & Mackauer (1992) who suggested that a female parasitoid must be able to lay the greatest number of eggs in the first oviposition opportunity to ensure the success of the offspring.

Host mortality without offspring production may be related to the significant reduction of eggs in the female ovary after the first oviposition. Females decreased the quantity of eggs accessible for additional host parasitism in a brief recovery time following the first oviposition, suggesting that many eggs are placed at once. Therefore, these results support the hypothesis that, after a successful parasitism of a sugarcane borer pupa, there will be restrictions on the availability of eggs for a host attack, at least for a resting period of 24 hours. Regardless, we cannot rule out the possibility that a female *T. howardi* abandoning a parasitized host is incapable of killing another host. In fact, pupae were killed with or without the emergence of the parasitoids, a result presumably caused by the female that had already parasitized a host and carried few eggs to address a subsequent oviposition. The death of the host without producing offspring indicates that the number of parasitoid larvae developing in the host was insufficient to overcome the host's immune system.

Eulophid parasitoids that attack large pupae need to lay multiple eggs or a lot of eggs at once to overcome the host's defenses (Andrade *et al.* 2010, Pereira *et al.* 2017), and to become parasites, they might stay with the host (Matthews *et al.* 2009). The results of this study showed that *T. howardi* females abandon the parasitized sugarcane borer pupa lodged in the stem, contradicting the hypothesis that the female would remain with its large host. Based on our data, an oviposition of *T. howardi* on a pupa of *D. saccharalis* resulted in the production of several young (up to 70 young on average), leaving only a few eggs in the ovary. In fact, using *T. molitor* pupae to rear *T. howardi* requires its exploitation by several parasitoid females to successfully kill the host and produce a greater number of offspring (ca. 130–140 per pupa on average) than would be produced by just a female. This indicates that more than one female laid eggs in a single *T. molitor* pupa compared to a *D. saccharalis* pupa (ca. 40–70 offspring). Thus, parasitized pupae of *D. saccharalis* are abandoned because one oviposition is enough to kill them and produce viable offspring.

Despite the fact that the parasitoid did not remain with the sugarcane borer parasitized pupa inside the stalk, the average number of offspring was 137 parasitoids, with a maximum of

237 parasitoids produced per pupa, a number greater than the average number of descendants produced by a female performing a single oviposition. These findings suggest that under laboratory settings, more than one parasitoid female found the pupa inside the stalk, resulting in superparasitism. This behavior has been seen both in the wild and in the laboratory (Janssen 1989, Matthews & Deyrup 2007, Pereira *et al.* 2017). Although superparasitism was formerly thought to be an adaptive mistake, it now enhances natural selection by promoting competition among individuals of the same species (Bakker *et al.* 1985). Superparasitism in a melonworm pupa boosted the number of progeny and its fitness, according to research with the eulophid *Palmistichus elaeisis* Delvare & LaSalle (Hymenoptera: Eulophidae) (Pereira *et al.* 2017). This might explain the behavior of *T. howardi* leaving the parasitized host pupa and the superparasitism, confirming its suitability as a host.

In summary, *T. howardi* can be reared using *T. molitor* pupae for up to 30 generations without losing its preference and performance over *D. saccharalis* pupae. Regardless of the natal host, *T. molitor* or *D. saccharalis*, parasitoid females produced few mature eggs within 24 hours of maturity and only attained maximum egg load between 72 and 96 hours. Females of *T. howardi* lay the majority of their eggs in a single oviposition, and the first oviposition is sufficient for *D. saccharalis* parasitism. The number of eggs remaining in the ovary after the first oviposition is significantly reduced, and egg load recovery does not occur within 24 hours of resting. Fresh parasitism of *T. howardi* on a second pupa of *D. saccharalis* will cause the pupa to die; however, this parasitism has a reduced chance of producing progeny. Furthermore, following *D. saccharalis* pupa parasitism inside the sugarcane stalk, the parasitoid females abandon their host and disperse in the field corroborating that one virgin female and one oviposition is enough. However, other females may also find a parasitized pupa inside the stalk and superparasitize it.

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Table 1. Biological characteristics of *Tetrastichus howardi* progeny obtained at different generations parasitizing *Diatraea saccharalis* pupae after continuous rearing using the alternative host *Tenebrio molitor*.

Generations	Time for 1st-choice (min)	Handling time (min)	Parasitism (%)	No. of offspring	Sex ratio (%♀)
F0[1]	26.1 ± 7.07 b	183.6 ± 37.45	93.0 ± 6.0	55.5 ± 5.63	92.8 ± 0.90
F5	223.5 ± 43.03 a	201.6 ± 56.34	100	69.7 ± 7.44	93.3 ± 0.60
F15	193.9 ± 41.64 a	236.6 ± 38.86	100	52.3 ± 6.23	90.9 ± 0.70
F30	166.6 ± 50.87 a	221.4 ± 37.22	92.0 ± 8.0	45.1 ± 7.46	90.6 ± 2.10
Statistics	*F* = 4.27 *df* =3, 60 ***P* = 0.008**	*F* = 0.71 *df* = 3, 59 *P* = 0.55	χ^2 = 26.48 *df* = 3, 76 *P* = 0.57	*F* = 1.93 *df* =3, 45 *P* = 0.14	*F* =1.29 *df* = 3, 45 *P* = 0.29

[1] F0 stands for females emerged from *Diatraea saccharalis* pupae and offered pupae of *Diatraea saccharalis* (DD) for parasitism.

Table 2. Parasitism performance of *Tetrastichus howardi* females accomplishing sequential ovipositions upon *Diatraea saccharalis* pupae. Values between brackets refer to the raw numbers of pupae.

Sequential ovipositions	% of pupae parasitized[1]	% of mortality of attacked pupa	% of emergence progeny	No. offspring produced per pupa
1st	100* (31/31)	100^{ns} (31/31)	96.7* (30/31)	70.6 ± 10.3* (30 pupae)
2nd	48.4 (15/31)	93,3 (14/15)	35.7 (5/14)	38.0 ± 8.0 (5 pupae)
χ^2	9.25	0.11	14.84	4.99
P-value	0.0024	0.73	0.0001	0.025

[1] Asterisks indicate that better performance was obtained for first host oviposition.

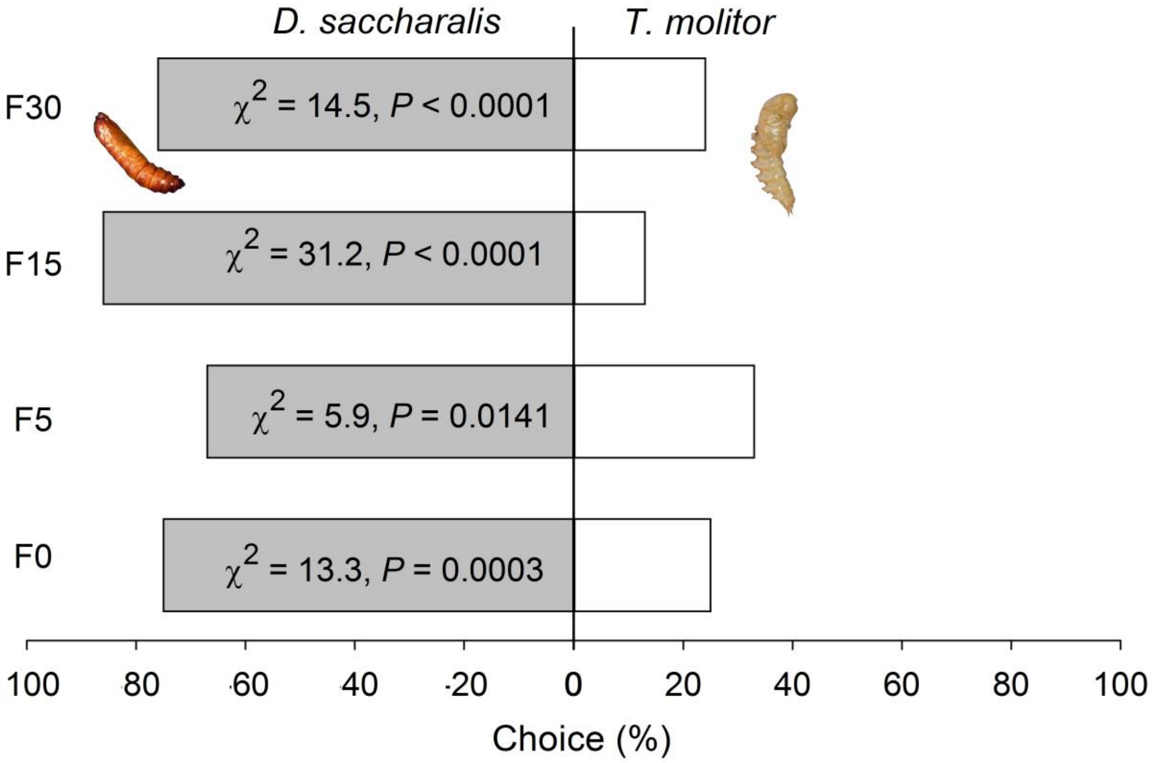

Figure 1. Females of *Tetrastichus howardi* were allowed to choose between pupae of *Diatraea saccharalis* or *Tenebrio molitor* after reared continuously with the alternative host *Tenebrio molitor* pupae during 30 generations. Bars represent the percentages of female choosing either pupae over an observation period of 10 hours. Chi-squares and respective p-values are given inside bars for pair-wise comparisons.

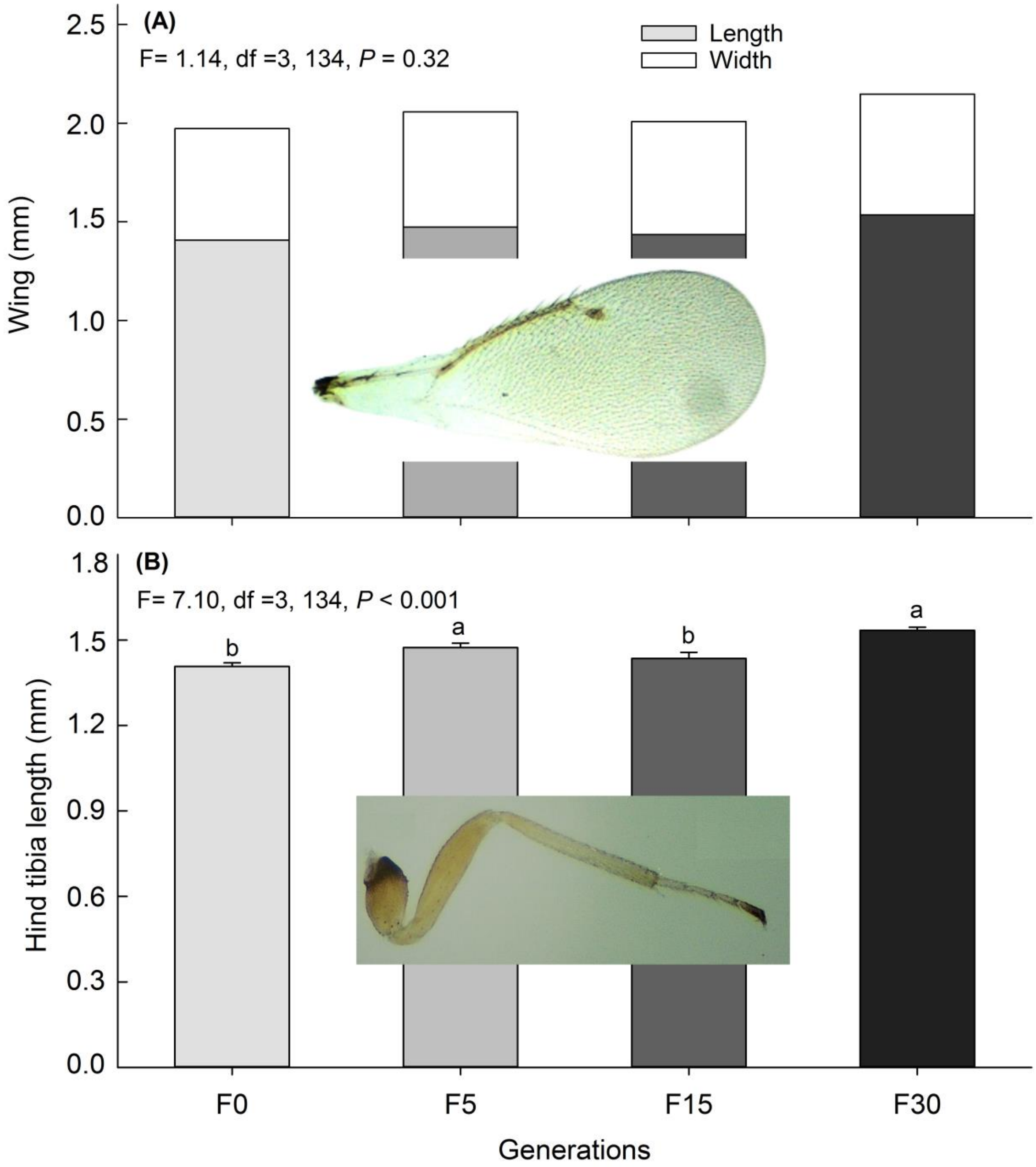

Figure 2. Size of forewing (A) and hindleg tibia (B) of *Tetrastichus howardi* females after different generations of continous rearings using pupae of *Tenebrio molitor*. Bars with different letters indicate statistical significance means compared by a contrast analysis ($P < 0.05$).

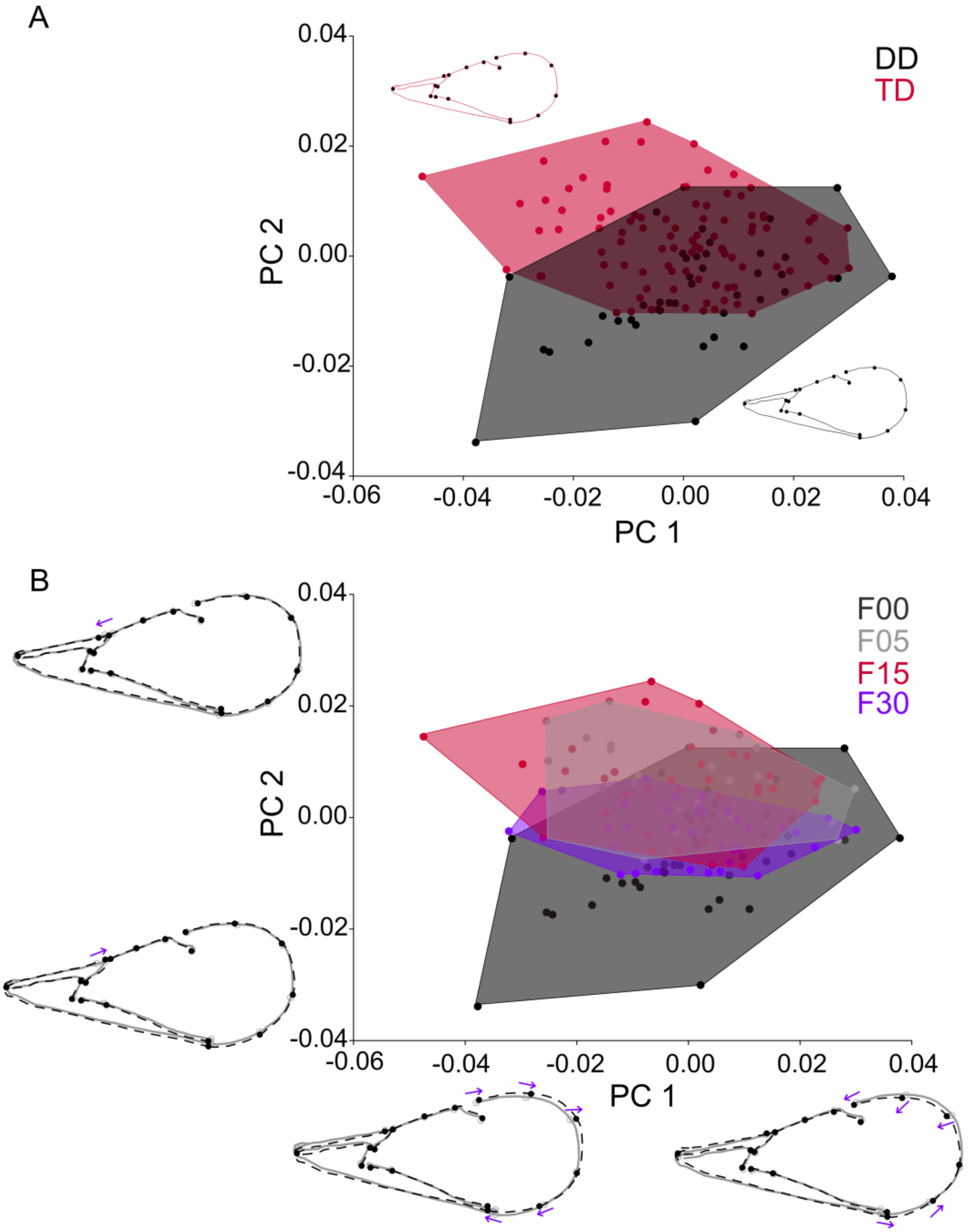

Figure 3. Representation of the Principal Component Analysis (PCA) for data of wing of *Tetrastichus howardi* females reared for 30 generations using the alternative host, *Tenebrio molitor*, as a function of host switch (A) and at each of 30 generations accomplished (B) captured by the PC1 and PC2 axes. Dotted lines with black dots (i.e. landmark) stand for changing on shape and gray line stand for the average of shape associated by the principal components (PC). Each point in the dispersion graphics represent the shape of the wing. Host switch from *Diatraea saccharalis* to *Diatraea saccharalis* (DD) at F00 generation, and from *Tenebrio molitor* to *Diatraea saccharalis* (TD) at F05, F15, and F30 generations.

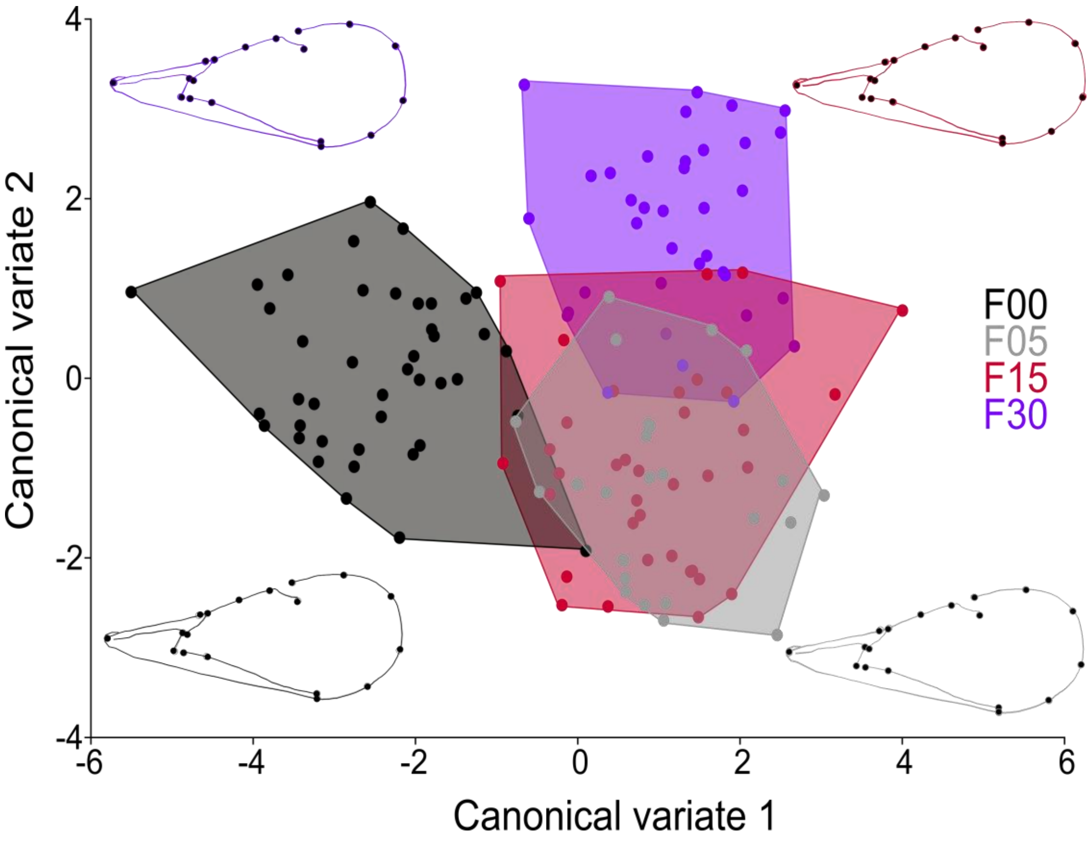

Figure 4. Canonical variate analysis (CVA). Differences in the average of wing shape generation of *Tetrastichus howardi* that were reared during 30 generations on alternative host *Tenebrio molitor*.

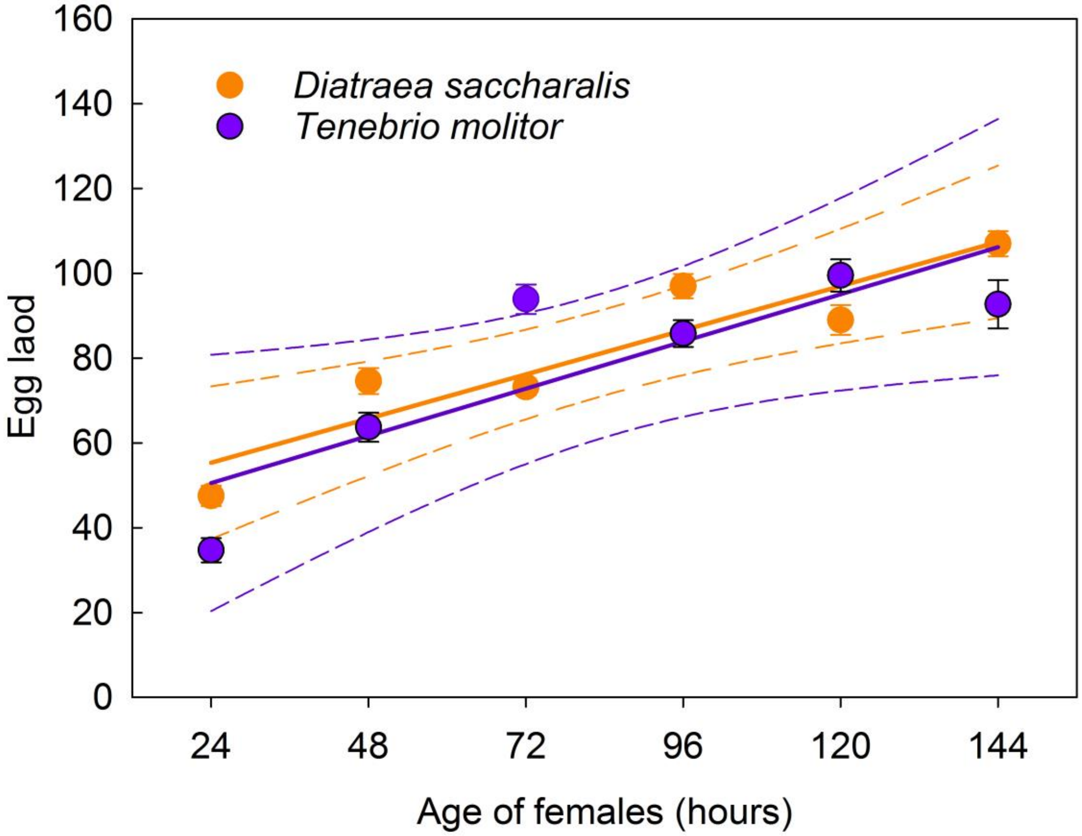

Figure 5. Egg loading by naive females of *Tetrastichus howardi* reared using the target host, *Diatraea saccharalis* ($y = 44.8 + 0.435x$, $r^2 = 0.72$, $F_{1, 58} = 144.62$, $P < 0.0001$) or the alternative host, *Tenebrio molitor* ($y = 39.45 + 0.46x$, $r^2 = 0.69$, $F_{1, 58} = 73.83$, $P < 0.0001$).

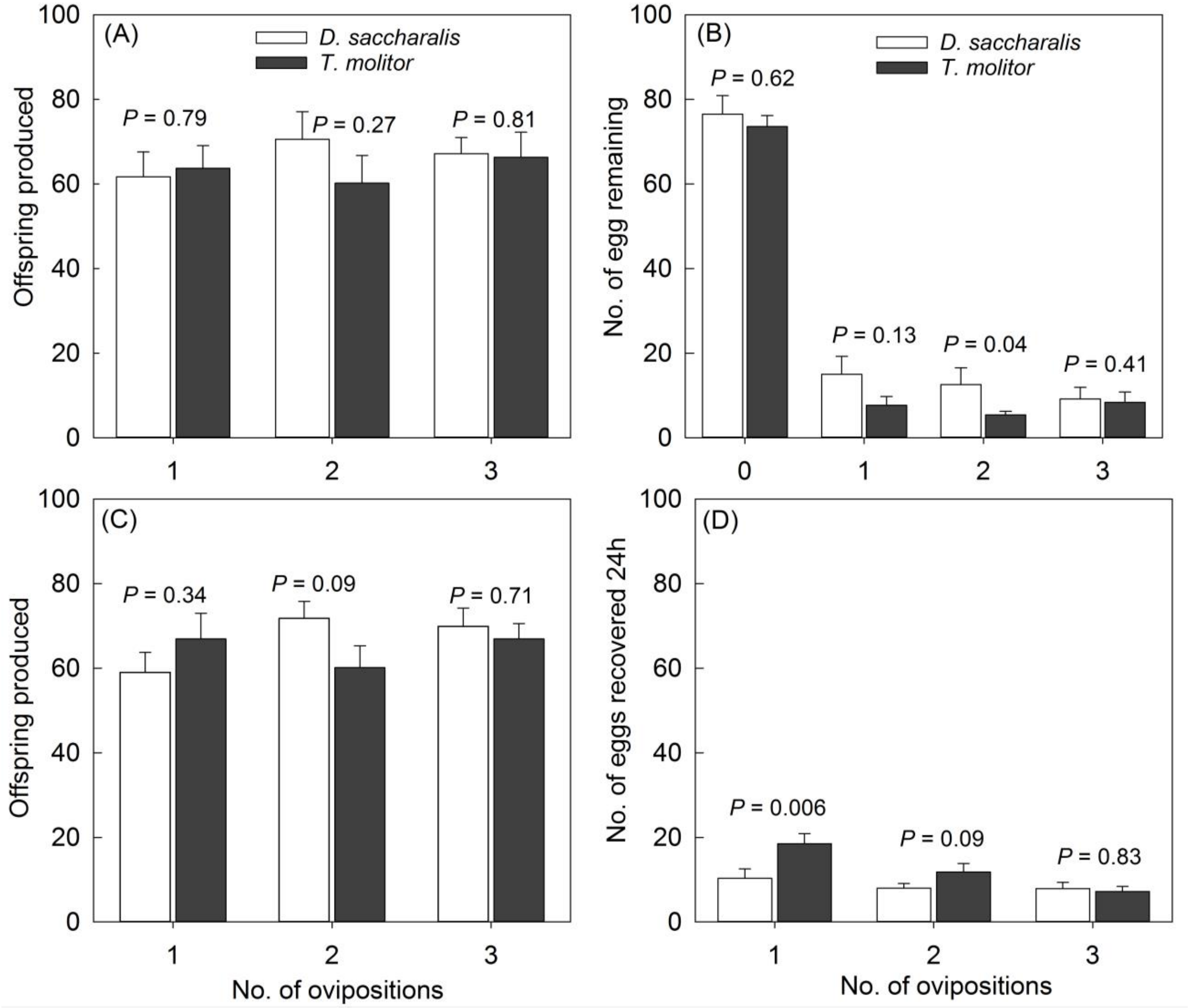

Figure 6. Mean number (+SE) of offspring produced per parasitized pupa of *Diatraea saccharalis* after one to three observed ovipositions in the same pupa by a single female *Tetrastichus howardi* over 12h-photophase period (A and C), and remainiong eggs in the ovary of these females dissected without oviposition (0) or right after completing one to three ovipositions (B) or after 24 hours-recovring period without oviposition (D). Nota: *P*-values stand for comparisons between parental hosts; see text for details of the statistics.

SUPPLEMENTARY MATERIAL

Table 1S. *P*-values from permutation tests (10 000 permutation rounds) for Mahalanobis and Procrustes distances amongst different generations of continuous rearing using pupae of the alternative host *Tenebrio molitor* offered pupae of *Diatraea saccharalis*. Mahalanobis (normal font) and Procrustes (*Italic font*). Significant differences: $P < 0.01$.

Generations	F0	F5	F15
F5	4.07, P < 0.0001 *0.01, P = 0.0011*	-	
F15	3.79, P < 0.0001 *0.01, P < 0.0001*	2.73, P <0.0001 *0.008, P=0.0903*	-
F30	4.02, P < 0.0001 *0.01, P = 0.0006*	3.24, P < 0.0001 *0.01, P = 0.0016*	2.93, P < 0.0001 *0.01, P = 0.0012*

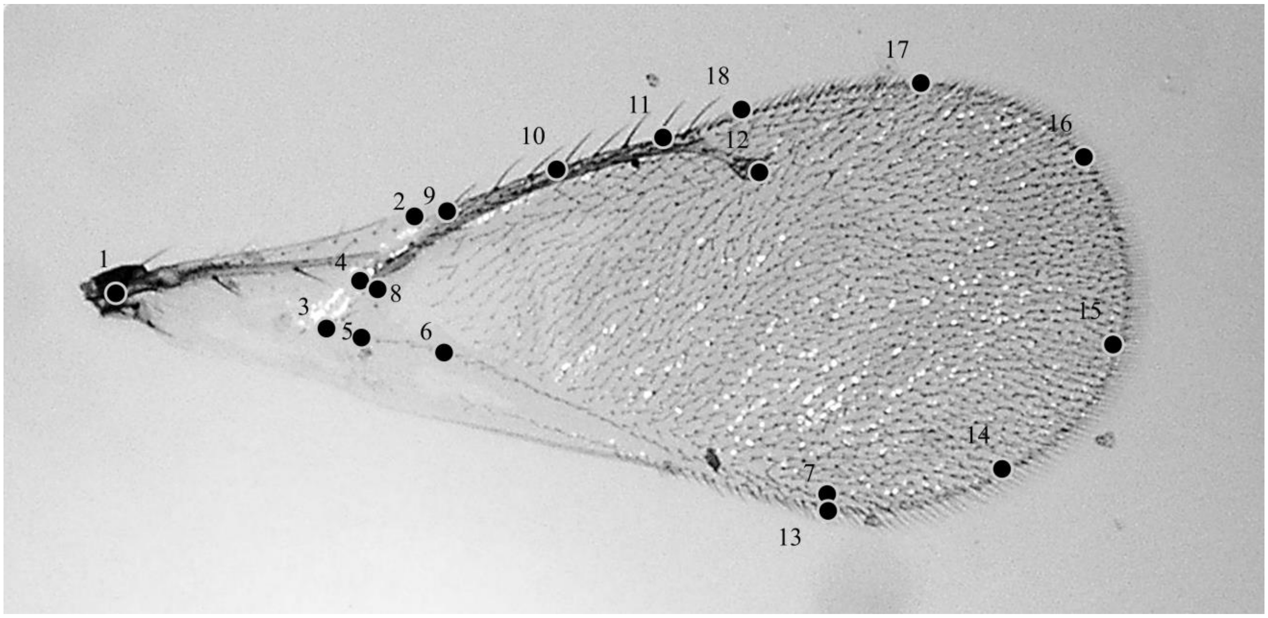

Figure 1S. Landmarks used in morphometric analysis of the forewing *Tetrastichus howardi* female.

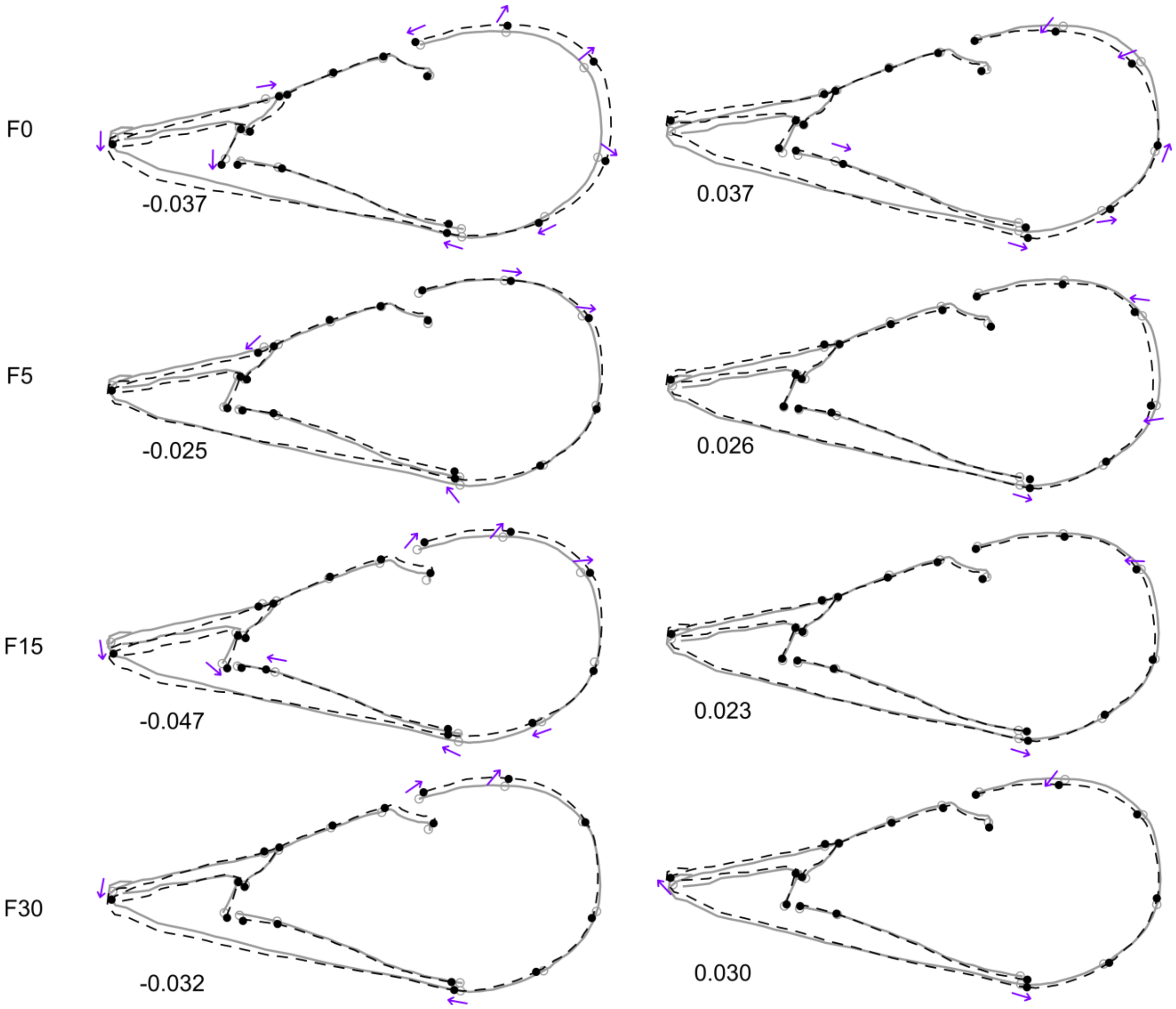

Figure 2S. Variation in the front wing shape of *Tetrastichus howardi* females assessed at F0, F5, F15, and F30 generation from 30 rearing generations using pupae of the alternative host, *Tenebrio molitor*. The left wings show changes in the shape portrayed by the lower negative extrem values and wings on the rigth show the changes in the shape portrayed by the higher positive extreme values at each generation. The dotted lines with black marks indicate the change in shape while the gray line indicate the average shape associated to PC1 on scale factor of 1.0

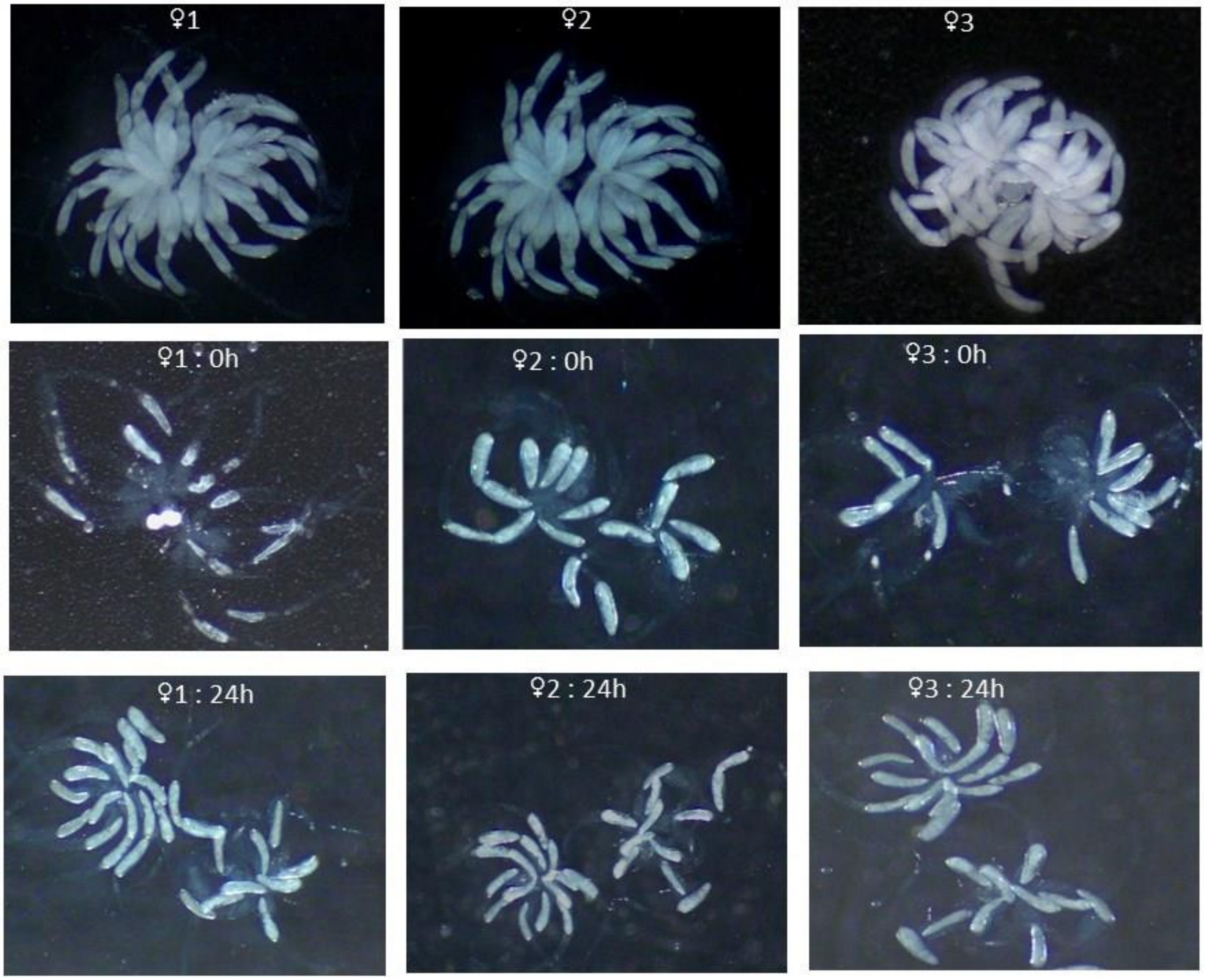

Figure 3S. Egg loading of *Tetrastichus howardi* as a function of ovipositions in randomly dissected females 96 hours old before oviposition (♀1-♀3), females right after accomplishing one oviposition (♀1: 0h - ♀3: 0h), and females after 24 hours of resting post the first oviposition (♀1: 24h - ♀3: 24h).

CAPÍTULO 3

BIOLOGICAL AND BEHAVIOURAL RESPONSES OF THE SUGARCANE BORER PARASITOID *Tetrastichus howardi* (OLLIFF) (HYMENOPTERA: EULOPHIDAE) TO INSECTICIDES

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[1]Bermúdez, N.C. D.V. Nascimento, R.P. Morato, C.S.A Silva-Torres & J.B. Torres. Biological and behavioural responses of the sugarcane borer parasitoid *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae) to insecticides. Journal of Applied Entomology. https://doi.org/10.1111/jen.13165.

ABSTRACT – The pupal parasitoid *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae) parasitizes pupae of sugarcane stalk borers. Parasitoids used for biological control of sugarcane stalk borers target eggs and larvae, and *T. howardi* will add mortality to the pest stage with high survival. Nevertheless, insecticide applications required to control sugarcane borers and other sugarcane pests can affect *T. howardi* parasitism. Survival, walking, evasion, and parasitism of *T. howardi* females were evaluated in response to the dry residue of insecticides from different chemical groups: chlorantraniliprole, bifenthrin, spinetoram, ethiprole, fipronil, and thiamethoxam. Parasitoid females were confined on sugarcane leaf containing residues of the insecticides at 2-, 24-, 48-, 72-, and 96-h intervals after application, survival monitored during the 96-h period with subsequent determination of parasitism in the laboratory. Furthermore, female walking behavior was observed individually using half-treated arenas with insecticides. Chlorantraniliprole was compatible with *T. howardi* irrespective of residual intervals and studied variables. Other tested insecticides did not allow parasitoid survival up to 96h of confinement on dry residues and, consequently, parasitism. Locomotory activity indicated that parasitoid females might avoid residues of spinetoram and ethiprole. Females released in cages containing spinetoram and ethiprole-treated sugarcane plants with the chance to move to untreated plants, parasitized pupae placed on either plant within a 24h-exposure period, which resulted in offspring production. Females observed for 10 min in a half-treated arena with chlorantraniliprole, ethiprole, fipronil, spinetoram, bifenthrin, or thiamethoxam exhibited 100%, 73.3%, 70%, 66.7%, 10%, and 0% survival after 24h, respectively. Chlorantraniliprole was compatible with *T. howardi* through the bioassays conducted, and parasitoids showed ability to parasitize their hosts on spinetoram- and ethiprole-treated plants moving away from the treated environment.

KEY WORDS: Insecticide selectivity, augmentative biological control, insect behaviour, sugarcane stalk borer

RESPOSTA BIOLÓGICA E COMPORTAMENTAL DO PARASITOIDE DA BROCA-DA-CANA *Tetrastichus howardi* (Olliff) (HYMENOPTERA: EULOPHIDAE) A INSETICIDAS

RESUMO – *Tetrastichus howardi* (Olliff) (Hymenoptera: Eulophidae) parasita pupas das brocas-do-colmo. Parasitoides utilizados para o controle da broca-do-colmo da cana controlam ovos e larvas, e *T. howardi* adicionará mortalidade ao estágio de pupa. As aplicações de inseticidas para controlar pragas da cana podem afetar o parasitismo de *T. howardi*. A sobrevivência de *T. howardi*, caminhamento, evasão e parasitismo foram avaliados em resposta ao resíduo seco dos inseticidas clorantraniliprole, bifentrina, espinetoram, etiprole, fipronil e tiametoxam. Fêmeas do parasitoide foram confinadas em folhas de cana contendo resíduos dos inseticidas nos intervalos de 2, 24, 48, 72 e 96h após aplicação, com a sobrevivência monitorada durante 96h, com posterior determinação do parasitismo, em laboratório. O comportamento das fêmeas foi observado em arenas semi-tratadas com inseticidas. Clorantraniliprole foi compatível com *T. howardi* independente do intervalo residual e variáveis estudadas. Os demais inseticidas não permitiram sobrevivência do parasitoide até 96h de confinamento em resíduos secos e, consequentemente, o parasitismo. A atividade locomotora indicou que as fêmeas podem evitar resíduos de espinetoram e etiprole. Fêmeas liberadas em gaiolas com plantas tratadas com ethiprole e espinetoram e com chance de escolha, parasitaram pupas em plantas tratadas e não tratadas, com produção de descendentes. Fêmeas observadas por 10 min em arena semi-tratada com clorantraniliprole, etiprole, fipronil, espinetoram, bifentrina ou tiametoxam exibiram 100%, 73,3%, 70%, 66,7%, 10% e 0% de sobrevivência após 24h, respectivamente. Clorantraniliprole foi compatível com *T. howardi* através dos bioensaios conduzidos, e os parasitoides mostraram capacidade de parasitar hospedeiros em plantas tratadas com etiprole e espinetoram e evadir da área tratada.

PALAVRAS-CHAVE: Seletividade de inseticidas, controle biológico aumentativo, comportamento de insetos, broca da cana-de-açúcar

Introduction

The warm weather in most Brazilian regions is favorable for sugarcane cultivation and offers availability of plants for infestations of different arthropod pests, causing yield loss. Along with Brazil, many other regions that produce sugarcane have to manage infestations by several species of stalk borers (Reay-Jones *et al.* 2005, Sallam 2006, Vargas *et al.* 2015). Among them, two are pests of sugarcane in Brazil: *Diatraea saccharalis* (Fabr.) and *Diatraea impersonatella* (=*flavipennella*) (Walker) (both Lepidoptera: Crambidae) (Freitas *et al.* 2007, Silva *et al.* 2014). Sugarcane borer larvae enter the sugarcane stalk, especially of the young plants, which may cause the death of the plants (dead heart). When they infest mature plants, the tunneled stalk sometimes breaks off, resulting in secondary aerial roots and new shoots. Besides these direct damages, indirectly, the stalks with holes made by larvae are colonized by a fungus (*Colletotrichum falcatum* Went) that reduces the factory gain (White *et al.* 2008, Franco *et al.* 2017).

The moths lay eggs in batches on leaves, and eggs remain exposed to mortality factors such as parasitism (Isas *et al.* 2016). Hatched larvae about one-week old bore into the stalk, where they complete development (≈40 to 45 days of larva and 8 to 10 days of pupa) (Holloway *et al.* 1928). Larvae developing inside stalks are parasitized by *Cotesia flavipes* (Cam.) (Hymenoptera: Braconidae) (Dinardo-Miranda *et al.* 2014). Despite the natural mortality caused by climatic factors, parasitism, and predation of sugarcane stalk borers in Brazil and elsewhere (Rossi & Fowler 2004, Fenoglio & Trumper 2007, Oliveira *et al.* 2012, Isas *et al.* 2016), insecticide applications are often required to restrain population growth and avoid economic losses. Currently, 65 commercial formulations of insecticides are available to spray sugarcane fields in Brazil against sugarcane stalk borers made of diamides, spinosyns, phenylpirazoles, neonicotinoids, pyrethroids, oxadiazine, IGRs, and others (AGROFIT 2022). Besides sugarcane borers, other different pest groups (spittlebugs, aphids, mealybugs, termites, weevils, etc.) may require insecticide applications of different modes of action. An estimate of insecticide applied in sugarcane crops in Brazil against insect pests is 4,758 tons, with a sale balance of US$262 million (Oliveira *et al.* 2014). Two to three insecticide applications have done during the first year of cropping, and between three and five applications are made on sugarcane fields in the following years due to the accumulation of biomass and occurrence of multiple pest species (sugarcane factory pest datasheet). These insecticides applications generate residues that may cause parasitoid mortality or alter their foraging and parasitism activities (Desneux *et al.* 2003, 2007, Fonseca *et al.* 2015, Guedes *et al.* 2016).

Despite of the availability of insecticide to recommend against stalk borers, applications target only young larvae before they enter the stalk (Fonseca *et al.* 2015, Wilson *et al.* 2022). Thus, additional control of sugarcane borer larvae and pupae inside the stalk is necessary. Applied biological control of larvae with *C. flavipes* has accomplished more than 3.5 million hectares treated out of ≈8.2 million hectares cultivated (Parra & Coelho Junior 2019). In areas with releases of *C. flavipes*, parasitism of sugarcane borer larvae varied from 13.2% to 42.8% (Dinardo-Miranda *et al.* 2014). Furthermore, the egg parasitoid *Trichogramma galloi* (Zucchi) (Hymenoptera: Trichogrammatidae) was released in 2 million hectares (Parra & Coelho Junior 2019). Nevertheless, there is a need for biological control agents to target pupae of stem bores, the last stage of the pest, before initiating a new generation. Mortality at any developmental stage reduces pest population growth, and mortality of early stages is caused by several mortality factors (i.e., natural enemies and non-natural enemies) (Peterson *et al.* 2009). On the other hand, additional mortality in the late developmental stage, such as "protected" pupae of the sugarcane borer, is expected to significantly influence its population density considering the high survival rate of pupae (Van Rensburgi *et al.* 1988, Mukunthan 1989). The parasitoid *Tetrastichus* *howardi* (Olliff) (Hymenoptera: Eulophidae) parasitizes pupae of *Diatraea* spp. (Cruz *et al.* 2011, Vargas *et al.* 2011). Barbosa *et al.* (2019) found an average of 39% parasitism of sentinel pupa placed in the field of sugarcane borer. Experimental releases of *T. howardi* obtained 1.9% to 62.5% parasitism of pupae at 20m and 4m from the release point, respectively, allowing the estimation of 102 parasitoid females to be released per sugarcane borer pupa (Barbosa *et al.* 2019). Currently, *T. howardi* is available for augmentative biological control (Biohowardi®). Therefore, *T. howardi* offers an option to control pupae of sugarcane stalk borers (Barbosa *et al.* 2019, Rodrigues *et al.* 2021, Pereira *et al.* 2021).

Tetrastichus howardi exhibits a series of successful traits as a biological control agent. Females parasitize the host pupae inside the sugarcane stalk (Kfir *et al.* 1993, Barbosa *et al.* 2019), fulfill development and accomplish parasitism when reared at temperature regimes between 18 and 34 °C (Yan *et al.* 2021). *Tetrastichus howardi* can be reared in insectaries using pupae of different species of lepidopteran (Barbosa *et al.* 2015, Piñeyro *et al.* 2016, Pereira *et al.* 2022) and pupae of the yellow mealworm *Tenebrio molitor* (L.) (Coleoptera: Tenebrionidae) (Vargas *et al.* 2011, Barbosa *et al.* 2019, Tiago *et al.* 2019), produced at low-cost diets. According to Machado *et al.* (unpublished data), one pupa of *T. molitor* generates an average of 130.5 parasitoids at ≈ 0.00022 US dollars per parasitoid produced, which allows the release of the 67,000 parasitoids recommended to manage a high infestation of sugarcane stalk borers at 17.78 US dollars per hectare. Furthermore, considering the small size of the adult parasitoid, large numbers of fed and mated females can be released using drones.

This study investigated the toxicity of dry residues of six insecticides to *T. howardi*. The insecticides chosen target stalk stem borers and other sugarcane pests. Parasitoid survival, parasitism, walking, and evasion behaviours were determined. We hypothesize that broad-spectrum insecticides will cause higher mortality rates and may impair parasitoid behavior. Alternatively, more specific compounds are expected to have less impact on parasitoid survival and behaviour.

Material and Methods

Insect population sources and rearing methods. A sugarcane borer colony, *D. saccharalis*, was established in the "Laboratório de Controle Biológico de Insetos" of the "Universidade Federal Rural de Pernambuco" (UFRPE), Recife, Pernambuco State, Brazil, using pupae donated by the Associação de Plantadores de Cana da Paraíba (ASPLAN), located in the district of Pitanga da Estrada, Mamanguape, PB, Brazil. The physical conditions were 22 ± 1 °C, 70 ± 10% of R.H., and a photoperiod of 12:12h L:D.

Larvae of *D. saccharalis* were reared using an artificial diet prepared after Hensley & Hommond (1968). The diet consisted of soybean meal, wheat germ, sugar, vitamin solution, Wesson salts, ascorbic acid, water, and anti-contaminants (Nipagin and antibiotics). The rearing procedures were according to Fonseca *et al.* (2015). Briefly, newly hatched larvae were transferred to vials (7.5 × 14 cm in diam × Ht) containing an artificial diet. After 30 days of rearing, larvae were transferred to plastic boxes (30 × 18 × 4 cm in L × W × Ht), where they received diet until pupation. Pupae were collected and placed in acrylic boxes (8.5 × 8.0 cm) lined with filter paper containing a moistened cotton pad until adult emergence. Adults were fed a 10% honey-water solution, offered in moistened cotton inside bottle caps placed on the bottom of the rearing cages, made of cylindrical tubes of PVC measuring 22 × 20 cm (Ht × diam) lined with paper for oviposition. Eggs collected were cleaned by immersion for 3 minutes in a solution made with 1% copper sulfate and 3% formalin, then rinsed with distilled water. Eggs were placed in Petri dishes (2 × 15 cm in Ht × diam) for 5–6 days to hatch, and then the larvae were used to initiate a new rearing cycle.

The yellow mealworm, *T. molitor*, has been maintained in the same laboratory since 2016. Larvae and adults are reared with a diet consisting of 97% wheat flour and 3% yeast, as described in Torres *et al.* (2006). Vegetables such as slices of carrots, sweet potatoes, or open slices of sugarcane stalks are offered over the diet. Pupae served for parasitoid rearing and colony maintenance.

The parasitoid, *T. howardi*, colony was established with parasitized *D. saccharalis* pupae donated by the Laboratório de Controle Biológico de Insetos (LECOBIOL), from the Universidade Federal da Grande Dourados (UFGD), Dourados, MS, Brazil. After emergence, adult parasitoids were transferred to glass tubes (10 × 2 cm in Ht × diam) at rate of seven females per vial, offered honey droplets as food and closed with plastic film. The rearing procedure for *T. howardi* followed Vargas *et al.* (2011) using *T. molitor* pupae (≤ 48h old). Each vial with parasitoid females received one *T. molitor* pupa for three days. After, the parental females were discarded, and the pupa reared until adult parasitoid emergence.

Plants and Insecticides. Sugarcane plants were grown in a greenhouse using plastic pots of 5L-volume-containing soil. Plants were used when 0.4 or 1.0 m tall on specific bioassays. They received insecticide applications to produce insecticide residues (see further description).

Six registered insecticides purchased from a local market were assayed using commercial formulations at the label rate recommended to spray sugarcane fields (AGROFIT 2022, Table 1). The selected insecticides represent different chemical classes and five modes of action (MoA), aiming to offer control against various sugarcane pests and insecticide rotation plans (Sparks & Nauen 2015). Insecticide treatments consisted of solutions prepared using the label rate diluted into tap water (pH 5.8–6.5) added 0.1% of the surfactant Haiten® (Arysta Lifescience do Brasil, Pirapora, SP, Brazil). Control treatments used only water and 0.1% surfactant solution. Insecticide solutions were applied to the drip point using a hand sprayer (Guarany^{TM}, Catanduva, SP) with adjustable pre-compression at 2.8 Kgf and a capacity of 1.25 litters.

Toxicity of Insecticide Residues to Adults of *Tetrastichus howardi*. Females of *T. howardi*, 24h-old and honey-fed, were used in the bioassays. In order to determine the toxicity of the selected insecticides to *T. howardi* three bioassays were conducted using insecticide-dry residues, aiming to evaluate: the lethal effect on adults confined with dry insecticide residues (bioassay *i*), parasitism by surviving females (bioassay *ii*), and the walking behaviour of parasitoid females (bioassay *iii*).

The bioassay *i* consisted of confining adult female parasitoids on dry residues of the insecticides obtained on sugarcane leaves. Leaves were harvested from insecticide-treated and untreated plants at 2-, 24-, 48-, 72-, and 96-h intervals after insecticide application and taken to the laboratory. Leaf pieces of 5 × 3 cm (L × W) were cut from the median portion of the leaves and placed covering the inner wall of glass vials (10 × 2 cm in Ht × diam) and closed with organdie fabric secured by a rubber band. Droplets of honey were offered on the leaf as food for parasitoids, stimulating them to walk on the treated leaf surface. The experimental design consisted of seven treatments (six insecticides and one untreated control group) with ten replicates per insecticide treatment. Each replicate consisted of 10 parasitoid females released per vial, except that 20 females were used in the chlorantraniliprole treatment. Further, five residual intervals (ca. 2-, 24-, 48-, 72-, and 96-h after insecticide application) and four evaluations of survival within each residual interval (ca. 24-, 48-, 72-, and 96-h after parasitoid confinement) were tested.

In the bioassay *ii* was assessed the parasitism rate of surviving females after contact with insecticide-dry residue from the bioassay *i*. Thus, females alive 96h after confinement at each of the five residual intervals were transferred to a clean vial at a rate of 7 females per vial and offered one pupa (24-h old) of *T. molitor* for parasitism. In this bioassay only treatments with surviving females were used: chlorantraniliprole and control groups. The parasitism rate, number of parasitoids emerging per parasitized pupa, and sex ratio of the parasitoids were recorded.

In the bioassay *iii*, the effect of the insecticide dry residue on an inert surface on the behaviour of the parasitoid female was evaluated. Glass plates (2 × 12 cm in Ht × diam) were used as the observation arena, aiming to obtain information about the walking and avoidance behaviours (Spindola *et al.* 2013). The bottom of the plate and its lid had the respective halves treated with 1 mL of the insecticide dilutions and named as treated area (TA) and untreated area (UTA). The two halves of the plate had a middle line drawn outside. The insecticide was applied with a graduated pipette (100–1000 mL, Labmate® HTL, Corning HTL SA, Warszawa, Poland) starting in the middle line toward the plate border, holding the plate with some inclination to avoid applying insecticide toward the untreated half of the plate. After application the test solutions, the plate was allowed to air-dry for about two hours inside an exhaust chamber Nalgon® 3700 (Nalgon Equipamentos Científicos, Itupeva, SP, Brazil). The control plate consisted of both halves treated only with water plus 0.1% surfactant.

Adult females of *T. howardi*, 48h-old, mated and honey-fed, were individually released in the arena and observed for 10 minutes. First, the females were kept separated in clean plate similar to the test arena to adapt to the glass surface, space, and light before releasing in the experimental arena. After, these females were individually captured into small vials (7.5 × 1 cm in Ht × diam) and transferred to the test arena to begin the observations. The female was released on a cover glass slip (1 × 1 cm) laid in the center of the plate between the treated and untreated halves, named the releasing zone. Each female was monitored for 10 minutes. Residency time on each half of the plate and the number of times that the female moved between insecticide-treated and untreated areas were recorded. This last measurement assessed the parasitoid's ability to detect the insecticide after contacting its residue and respond by increasing or decreasing the movement between the TA and UTA halves of the arena. Each plate was used to observe up to five females. At the end of the observation period, the female was transferred to a clean glass vial, and the mortality was recorded after 24h. Each insecticide treatment was tested with 30 females (replicates), while 20 females were observed for the control treatment. Observations were done during the photophase between 8 a.m. to 5 p.m. and at 25 ± 1 ºC.

Survival and Parasitism of *Tetrastichus howardi* with Chance of Evading from Spinetoram- and Ethiprole-Treated Environment. In this bioassay, we investigated how *T. howardi* females could avoid insecticide-treated areas while parasitizing the sugarcane borer. The insecticides tested were spinetoram and ethiprole, to which the parasitoid showed some avoidance in the bioassay *iii*. Plexiglas cages (50 × 40 × 50 cm L × W × Ht) were mounted in pairs by connecting them using a 35 × 14.5 cm L × diam tube made from transparent acetate sheets. The other three sides of the cages had openings of 15 cm in diameter covered by an anti-aphid screen to allow ventilation inside. Sugarcane plants 40 cm tall with 4–5 leaves were treated with spinetoram or ethiprole as done in bioassay *i*. After two hours of spraying, the plants were transferred to the laboratory. Treated and untreated plants were harvested and kept with their stems dipped into water in an 80-mL vial secured with cotton. One *D. saccharalis* pupa, 48h-old, was placed among the top leaves (whorl) of each treated and untreated plant as a host to assess parasitism activity. Thirteen and ten pairs of cages (replications) were performed for spinetoram and ethiprole, respectively, with corresponding numbers of 650 and 500 parasitoid females observed. Each insecticide was tested separately due to the logistics and number of cages used. Parasitoid females, 48h-old, mated, and honey-fed, were separated in glass tubes (10 × 2 cm in Ht × diam) at a rate of 50 females per tube. The tube was placed on the bottom of the cage containing the insecticide-treated plant and opened, allowing the parasitoids to leave. After 24h of releasing the females, the number of individuals found dead on the bottom of the cage was recorded. Furthermore, the number of parasitoids alive was tallied, and *D. saccharalis* pupae were transferred to glass tubes, where they were monitored for parasitoid or sugarcane borer emergence.

Statistical Analysis. The survival curves for adult parasitoids confined with insecticide residues were calculated (bioassay *i*). The mortality data (1 dead, 0 alive) censored across the four days of confinement (24-, 48-, 72-, and 96-h) with dry insecticide residues for each residual interval (2-, 24-, 48-, 72-, and 96-h after application) were submitted to the Kaplan-Meier method. Further, the survival curves were compared among insecticides by the Log-Rank test ($\alpha = 0.05$) using the software SigmaPlot 12.5 (Systat Software Inc. 2013).

The frequency analysis [Proc Freq of SAS (SAS Institute 2001)] was applied to test the hypothesis of an equal rate of parasitism (emerging parasitoids as a function of the original number of pupae offered to parasitism), and offspring sex ratio (proportion of females) between females that survived contact with chlorantraniliprole and the control group (bioassay *ii*). Furthermore, the number of offspring produced per parasitized pupa was submitted to two-way ANOVA 2×5 (chlorantraniliprole and control groups and five residual intervals) through Proc GLM of SAS (SAS Institute 2001). The means were compared by Fisher's test between treatments (df = 1) at each residual interval ($\alpha = 0.05$), and the difference among residual intervals within each treatment group was determined by the Tukey HSD test ($\alpha = 0.01$ after correction of α/no. of means in comparison).

Data on parasitoid walking behavior (bioassay *iii*), determined as the residence time for parasitoid females, were compared between the halves of the plate containing residues of insecticides by a paired *t*-test (Proc Means of SAS) under the hypothesis of equal time of parasitoid foraging in both areas. In addition, the irritability as an indicator of insecticide effect on parasitoid foraging behaviour was calculated considering that the insecticide caused irritability when the female parasitoid spent more than 50% of the time (> 5 out of 10 minutes of observation) in the untreated half of the plate. The outcome for an individual female was ranked as the insecticide causing irritability (score = 1) when the outcome of the difference between the time spent in the untreated-half and treated-half of the plate was greater than 1 (difference > 1), and lack of irritability (score = 0) when the difference is lower than 1 (difference < 1). Following the rank of scores, the percentage of females exhibiting irritability was determined for each insecticide tested. Further, the scores were submitted to a non-parametric analysis (Proc NPAR1WAY) and the outcome was compared between halves of the plate by the Wilcoxon's test (α = 0.05). The number of times that each female crossed between treated and untreated areas of the plate was compared among treatments using the non-parametric Kruskal-Wallis test, and the survival of these females, tallied 24h after testing, was analyzed by ANOVA with means compared by Waller-Duncan (α = 0.007 after correction for α = 0.05/no. of means in comparison). Finally, the number of parasitized pupae of the sugarcane borer and dead and alive parasitoids in the cages containing treated or untreated sugarcane plants were used to calculate the percentage of parasitism, percentage of parasitoid mortality, and survival. Due to the lack of error independence (parasitoids were released only in the cage containing treated plants), the means and their respective 95% confidence intervals were estimated and used to infer about the ability of the parasitoid females to parasitize and to evade insecticide-treated areas.

Results

Toxicity of Insecticide Residues to Adults of *Tetrastichus howardi*. *Lethal effect on adults confined with dry insecticide residues (bioassay i).* Adult survival was reduced over time as a function of tested insecticides and residual intervals (Fig. 1: 2h to 96h). Spinetoram, bifenthrin, ethiprole, thiamethoxam, and fipronil did not allow *T. howardi* survival 24h after confinement on leaves from the residual interval 2h after application. Survival of parasitoids confined on chlorantraniliprole residues was similar to that of those in the control group up to 72h, with 73.5% survival at the last evaluation time (Fig. 1: 2h). In the residual interval of 24h after application, parasitoid survival was similar between the chlorantraniliprole and control groups in all evaluation times, whereas bifenthrin and spinetoram allowed 29% and 7%, and 14% and 2% survival at confinement times 24h and 48h, respectively (Fig. 1: 24h residual time). Thiamethoxam, fipronil, and ethiprole caused 100% mortality.

In the 48-hour residual interval after insecticide application, parasitoid survival was similar between the chlorantraniliprole and control groups in all confinement times. In contrast, ethiprole and fipronil did not allow parasitoid survival 24h after confinement. Bifenthrin, spinetoram, and thiamethoxam allowed 50%, 48.5%, and 7% survival at 24h after confinement, respectively. The survival rate with these insecticides continued decreasing over time, without survival 96h after confinement (Fig. 1: 48h residual time).

In the subsequent residual intervals (72h and 96h after application) (Fig. 1: 72 and 96h), fipronil and ethiprole sustained high toxicity without parasitoid survival 24h after confinement. Spinetoram and bifenthrin allowed survival within 48h and 72h of confinement; and chlorantraniliprole allowed similar parasitoid survival to the control group.

Parasitism by surviving females (bioassay ii). Females of *T. howardi* that survived 96h confinement in the control and chlorantraniliprole groups showed similar parasitism rates across the residual intervals (control group: 40–70% parasitism; $\chi^2 = 2.29$, df = 4, $P = 0.68$; chlorantraniliprole: 60–80% parasitism; $\chi^2 = 7.21$, df = 4, $P = 0.12$). Likewise, the parasitism rates were also similar between females from the control group and those surviving exposure to dry residue of chlorantraniliprole for all residual intervals studied ($\chi^2 = 0.33$ to 2.02, df = 1, $P = 0.15$ to 0.56).

Despite the similar parasitism rate, the number of parasitoids produced per pupa differed between females from chlorantraniliprole and control groups ($F_{1,\ 36}$ = 5.93, P = 0.02). Likewise, there was a difference in the number of parasitoids emerging per pupa among the residual intervals ($F_{4,\ 36}$ = 3.29, P = 0.02), but not for the interaction between treatments and residual intervals ($F_{4,\ 36}$ = 1.76, P = 0.16). Females from the control and chlorantraniliprole groups produced, on average, 136.3 and 110.5 parasitoids per pupa, respectively, irrespective of the residual intervals. Furthermore, the number of parasitoids produced per pupa decreased as a function of insecticide residual intervals, except in the 72h residual interval after application. A greater number of parasitoids were observed for females in the control group than in chlorantraniliprole that survived the residual intervals of 2h ($F_{1,\ 5}$ = 43.07, P = 0.009) and 24h ($F_{1,\ 6}$ = 7.53, P = 0.03) (Fig. 2). This variation was responsible for the interaction effect between treatment and residual intervals regarding offspring production ($F_{4,\ 11}$ = 7.64, P = 0.003), due to greater emergence from pupae parasitized by females surviving in the residual interval 2h (Fig. 2). On the other hand, pupae parasitized by females that survived the contact with chlorantraniliprole produced a similar number of parasitoids across all residual intervals ($F_{4,\ 25}$ = 1.08, P = 0.38) (Fig. 2). The sex ratio of the parasitoids that emerged (proportion of females) stayed between 88.6% and 93.4% without difference between treatments or residual intervals (χ^2 = 3.66, df = 9, P = 0.13). Pupae that did not produce adult hosts or parasitoids were dissected to verify the parasitism. Thus, pupae from the chlorantraniliprole treatment showed 47.3% with mummified parasitoids inside. On the other hand, all parasitized pupae from the control group produced adult parasitoids.

Locomotory behaviour of parasitoid females exposed to dry insecticide residues (iii). Residues of spinetoram, ethiprole, and thiamethoxam affected the time spent by the parasitoid between the insecticide-treated and untreated halves of the plate (Fig. 3). Females of the parasitoid spent more time in the untreated half of the plate when testing spinetoram and ethiprole (429.9 ± 34.88 and 410.8 ± 33.54 sec, respectively). The opposite was observed with thiamethoxam, where the parasitoid female spent more time in the insecticide-treated half of the plate (342.9 ± 20.88 sec) than in the untreated-half (228.6 ± 20.92 sec). For bifenthrin, fipronil, chlorantraniliprole, and the control, parasitoid females did not show a difference in time spent between the insecticide-treated and untreated halves of the plate ($P > 0.05$) (Fig. 3).

The irritability was observed in parasitoid females exposed to spinetoram ($\chi^2 = 21.24$, $P < 0.0001$) and ethiprole ($\chi^2 = 15.25$, $P < 0.0001$), with females spending 80% and 75.8% of the time in the untreated half of the plate, respectively. On the other hand, females exposed to bifenthrin ($\chi^2 = 9.48$, $P = 0.002$) and thiamethoxam ($\chi^2 = 9.44$, $P = 0.002$) spent 70% and 73% of time in the insecticide-treated half of the plate, respectively. Females exposed to chlorantraniliprole, fipronil, and the control group showed similar time spent in both insecticide-treated and untreated halves of the plate (Fig. 4).

The number of times that each female moved between treated and untreated areas differed among treatments ($F_{6, 193} = 25.44$, $P < 0.0001$). The results characterized three groups of responses. The first group was formed by females exposed to chlorantraniliprole and thiamethoxam, with a greater number of crosses between the treated and untreated halves of the plate (13.7 ± 1.31 and 11.6 ± 0.91 times, respectively). The second group was formed by bifenthrin and control (7.1 ± 1.57 and 5.7 ± 0.53 times, respectively), while the third group was formed by fipronil, ethiprole, and spinetoram, with the lower number of changes between the untreated and treated halves of the plate (3.9 ± 0.53, 3.7 ± 0.47, and 3.7 ± 0.57 times, respectively) (Fig. 5).

Female survival 24h after the 10-min observation in the insecticide-half-treated plate and the control differed among treatments (Fig. 6). Females in the half-treated plate with chlorantraniliprole and the control group showed 100% survival. In addition, survival was greater than 66% for females exposed to half-treated plates with spinetoram, fipronil, and ethiprole. Females subjected to observation in the half-treated plate with bifenthrin and thiamethoxam exhibited 10% and 0% survival, respectively.

Survival and Parasitism of *Tetrastichus howardi* with Chance of Evading from Spinetoram- and Ethiprole-Treated Environment. After 24h of parasitoid release in the cage containing spinetoram-treated sugarcane plants, 86.5% (562 out of 650 released parasitoids) were found dead in the cage bottom. In addition, 9.2% (60 out of 650 parasitoids) contacted the insecticide residue on the plant and moved to and died in the paired cages containing untreated sugarcane plants (Fig. 7). The overall survival 24h after releasing the parasitoids was 4.3% (28 out of 650 parasitoids). Among the survivors, 39.3% and 60.7% were recorded in the cages containing treated and untreated plants, respectively. In addition, there was parasitism of 61.5% and 23.1% of the pupae on treated and untreated-plants, respectively. These parasitized pupae produced, on average, 127 and 178 parasitoids per pupa parasitized.

Female parasitoid parasitized pupae of the sugarcane borer and produced offspring when released on ethiprole-treated and untreated plants, despite the observed mortality. Female parasitoid exhibited 95.8% mortality when released inside the cages containing sugarcane plants treated with ethiprole (Fig. 7). Three out of 500 females were alive in the cages with ethiprole-treated sugarcane plants 24h after release. Despite that, 80% of pupae exposed on ethiprole-treated plants were parasitized and produced 226.1 offspring per pupa. Furthermore, 3.2% of the females (16 out of 500) had contact with ethiprole-treated plants, moved, and died in the paired cage containing untreated plants. Pupae exposed to parasitism on untreated plants was 70% and produced 227 offspring per pupa, on average (Fig. 7).

Discussion

Among the six tested insecticides with different modes of action to control sugarcane pests, only chlorantraniliprole was compatible with the parasitoid *T. howardi*. The parasitism of *T. howardi* can be significantly impaired when in contact with residues of bifenthrin, thiamethoxam, ethiprole, spinetoram, and fipronil deposited on the sugarcane foliage. Furthermore, these insecticides are registered to be used against different pests in sugarcane and other crops (AGROFIT 2022), extending their incompatibility with *T. howardi* and reducing the chances of its conservation within an IPM program.

Chlorantraniliprole showed compatibility with *T. howardi,* allowing survival between 70% and 100%, offspring production, locomotory, and a lack of irritability in contact with dry residues. Furthermore, the parasitoid exhibited a lack of behavioural changes between chlorantraniliprole-treated and untreated areas, suggesting that the parasitoid retained its foraging capability. Chlorantraniliprole belongs to the diamide insecticide class that acts selectively as a modulator of the ryanodine receptor in insects with primary activity against lepidopteran larvae (Sparks & Nauen 2015) and contamination of target pests predominantly through ingestion and contact with residues from an application on leaves (http://www.irac-online.org/). Currently, in Brazil, there are 16 commercial formulations made with chlorantraniliprole, eight in a single formulation and eight in mixtures ready-to-use with other insecticides (AGROFIT 2022). This opens the option for natural enemy conservation within IPM programs needing chlorantraniliprole applications. It is worth highlighting, however, that chlorantraniliprole in a mixture with other insecticides, mainly pyrethroids (e.g., lambda-cyhalothrin) and neonicotinoids (e.g., thiamethoxam), will lose its property of specificity due to the broad-spectrum action of the insecticides used in the mixture (Barbosa *et al.* 2017, Barros *et al.* 2018).

Females of *T. howardi* exposed to dry residues of chlorantraniliprole obtained on sugarcane leaves 2 and 24h after application showed some reduction in progeny production compared to females in the control group, which suggests a sublethal effect caused by insecticide-fresh residues. Chlorantraniliprole in the residual intervals of 48 and 96h did not affect survival rate, parasitism rate, or offspring production. Sublethal effects were found with the egg parasitoid *Trichogramma brassicae* (Bezdenko) (Hymenoptera: Trichogrammatidae), with a lower emergence rate, generational time, and adult longevity when exposed to chlorantraniliprole (Parsaeyan *et al.* 2020). The proportion of females in the progeny of *C. flavipes* exposed to chlorantraniliprole was also reduced when parasitizing the sugarcane stalk borer (Matioli *et al.* 2019). Despite that, in both studies and in this one, adult parasitoid survival was not affected. The findings corroborate the results of null or low impact of chlorantraniliprole upon different natural enemy species, including parasitoids (Gradish *et al.* 2011, Huang *et al.* 2011, Goulart *et al.* 2012, Muslim *et al.* 2018, Barros *et al.* 2018) and predator species (Barros *et al.* 2018, Machado *et al.* 2019, Potin *et al.* 2022, Morato *et al.* 2023).

In contrast to chlorantraniliprole, the other tested insecticides fipronil, thiamethoxam, bifenthrin, ethiprole, and spinetoram were toxic to *T. howardi* adult. Fipronil, bifenthrin, and thiamethoxam are considered non-selective insecticides for parasitoids and did not show a reduction in their toxicity with later residual intervals. Ethiprole is a phenylpyrazole like fipronil but has shown selectivity for certain natural enemies, such as the predatory ring-legged earwig, *Euborellia annulipes* (Lucas) (Dermaptera: Anisolabididae), and the boll weevil parasitoid, *Bracon vulgaris (*Ashmead) (Hymenoptera: Braconidae). The ring-legged earwig exhibited 98% survival when exposed to ethiprole residues 2h after application using the label rate recommended against boll weevil (Torres *et al.* 2021). Furthermore, *B. vulgaris* showed 52–80% survival when confined with dry residues of ethiprole obtained on treated cotton leaves

2h and 96h after application (Torres *et al.* 2021). Ethiprole showed a lower risk quotient (value = 4.85) for the egg parasitoid *Trichogramma evanescens* Westwood (Hymenoptera: Trichogrammatidae), compared to other phenylpyrazoles such as buteno-fipronil (811) and fipronil (321), when exposed to insecticide dry residues in glass vials (Wang *et al.* 2014). In the present study, ethiprole was maintained toxic to *T. howardi* through studied residual intervals up to 96h after application on sugarcane leaves. Nevertheless, females of *T. howardi* exhibited 60% survival 24h after being given the choice between ethiprole-treated and untreated halves of the arena and spent more time on the untreated half, indicating that the parasitoid can avoid ethiprole-treated surfaces. In fact, despite the high rate of acute toxicity, parasitoid females released in cages containing spinetoram- or ethiprole-treated plants parasitized the host pupae on sugarcane plants and produced offspring.

The residues of spinetoram and ethiprole are still toxic to *T. howardi* 96h after application under the studied confinement conditions. Mortality above 80% of boll weevils confined to leaves collected from field-treated plants was recorded 192h after the application of spinetoram (Rolim *et al.* 2019) or ethiprole (Torres *et al.* 2021), suggesting a long-lasting residual interval. Under field conditions, however, we can consider that the parasitoid can evade areas treated with these insecticides. When the parasitoid had contact with these insecticides in a half-treated arena, the results indicated an irritability effect, with the parasitoid surviving a short period of contact with the treated surface.

Spinetoram belongs to the spinosyn group of insecticides marketed to control lepidopteran larvae (Siebert *et al.* 2016) and shows compatibility with certain natural enemies (Srivastava *et al.* 2018, Costa *et al.* 2020). However, spinetoram was harmful to *T. howardi* with only 25% survival 96h after application. Similar to these findings, spinetoram was also toxic to other parasitoids (Hernandez *et al.* 2011). In addition, it seems that spinetoram is more harmful to parasitoids than predatory insects (Williams *et al.* 2003, Barros *et al.* 2018, Machado *et al.* 2019). Despite that, three outcomes can be described from the interaction between spinetoram and *T. howardi*. First, about 48.5% of females were alive 48h after confinement on dry residues of spinetoram. Second, over 60% of females survived short-time contact with spinetoram dry residues in treated plates resembling a short contact with treated plants when released; and third, parasitoid females can evade spinetoram-treated areas with host parasitism and production of descendants, favoring the conservation of the parasitoid in the field. Differently, the insecticides bifenthrin, fipronil, and thiamethoxam caused quick mortality of the parasitoid, precluding any chance of parasitoid maintenance. The ability of adult natural enemies to evade insecticide-treated areas may explain, in part, their presence in fields following the application of insecticides with high residual toxicity. Understanding the evasion response of the natural enemies to spinetoram and ethiprole might help their use in pest control program. For instance, the hymenopteran parasitoid *B. vulgaris* showed over 80% survival when confined to the dry residue of ethiprole, obtained 96h after application on cotton plants (Torres *et al.* 2021). Furthermore, ethiprole and spinetoram have been considered to increase recommendations for agricultural pest control (GVR 2022).

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Table 1. Insecticides commercial formulations, chemical groups, mode of action (MoA), the highest dose recommended to spray sugarcane crop from label, and target pests in sugarcane crop

Inseticides	Chemical group	MoA	Label dose per hectare	Target pests*
Bifenthrin 100 EC	Pyrethroid	3A	1200 mL	Ht, Pt
Chlorantraniliprole 350 WG	Diamide	28	450 g	Ht, Cl, Sl, Ds
Spinetoram 250 WG	Spinosyn	5	200 g	Ds
Ethiprole 200 SC	Phenylpyrazole	2B	1250 mL	Mf, Ht
Fipronil 800 WG	Phenylpyrazole	2B	500 g	Ds, No, Ht, Pt, Mf, Cc
Thiamethoxam 250 WG	Neonicotinoid	4A	1000 g	Ht, Mfry

*Ht, *Heterotermes tenuis*; Pt, *Procornitermes triacifer*; Cl, *Castnia licus*; Sl, *Sphenophorus levis*; Ds, *Diatraea saccharalis*; Mf, *Mahanarva fimbriolata*; Mfry, *Migdolus fryanus*; No, *Neocapritermes opacus*; and Cc, *Cornitermes cumulans*.

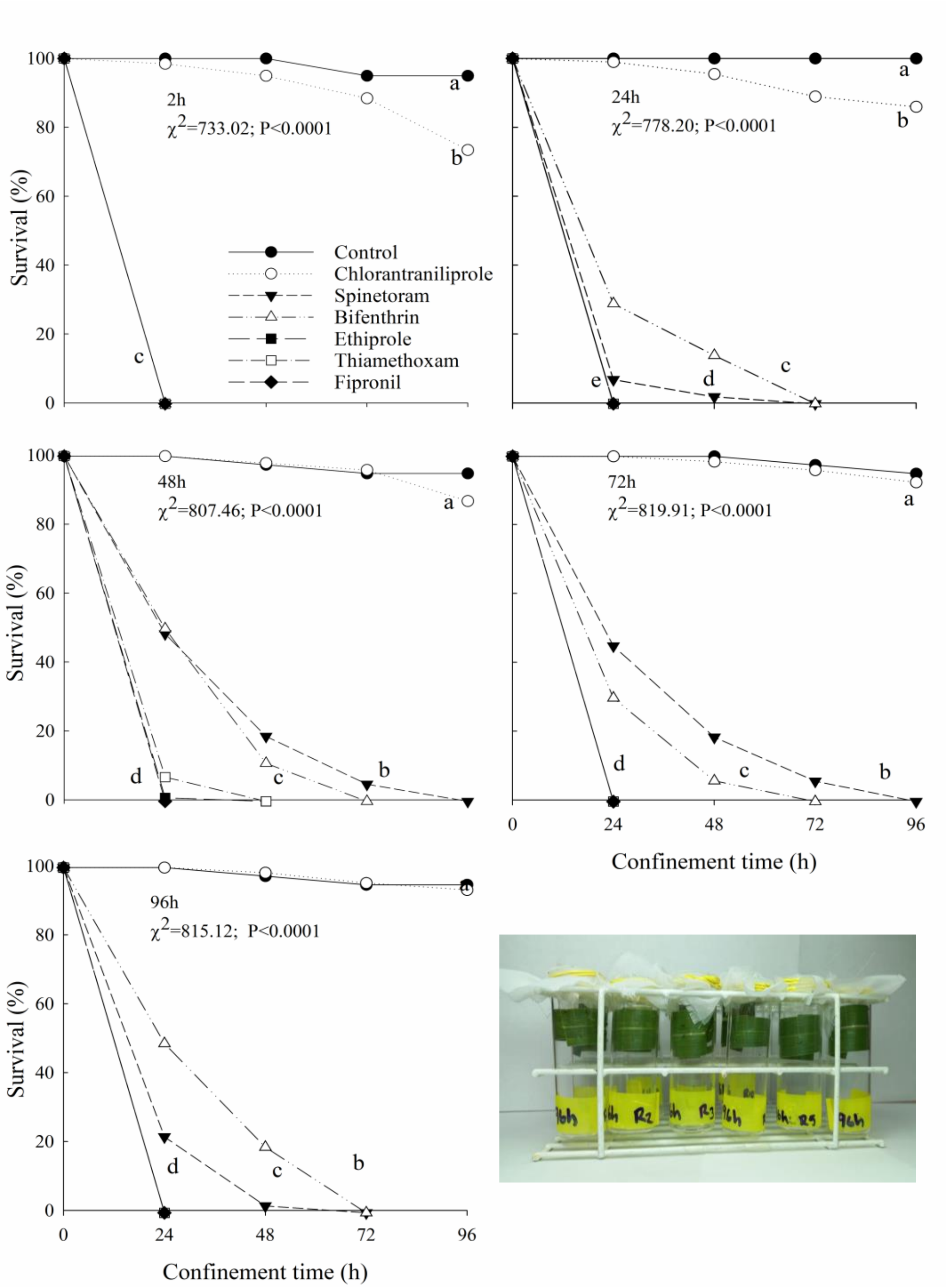

Figure 1. Survival of *Tetrastichus howardi* females confined for 96h on insecticide dry residues obtained on sugarcane leaves treated with different insecticides at residual intervals of 2h, 24h, 48h, 72h, and 96h after application in comparison to the control group. Survival curves compared by Log-Rank's test (df = 6, α = 0.05)

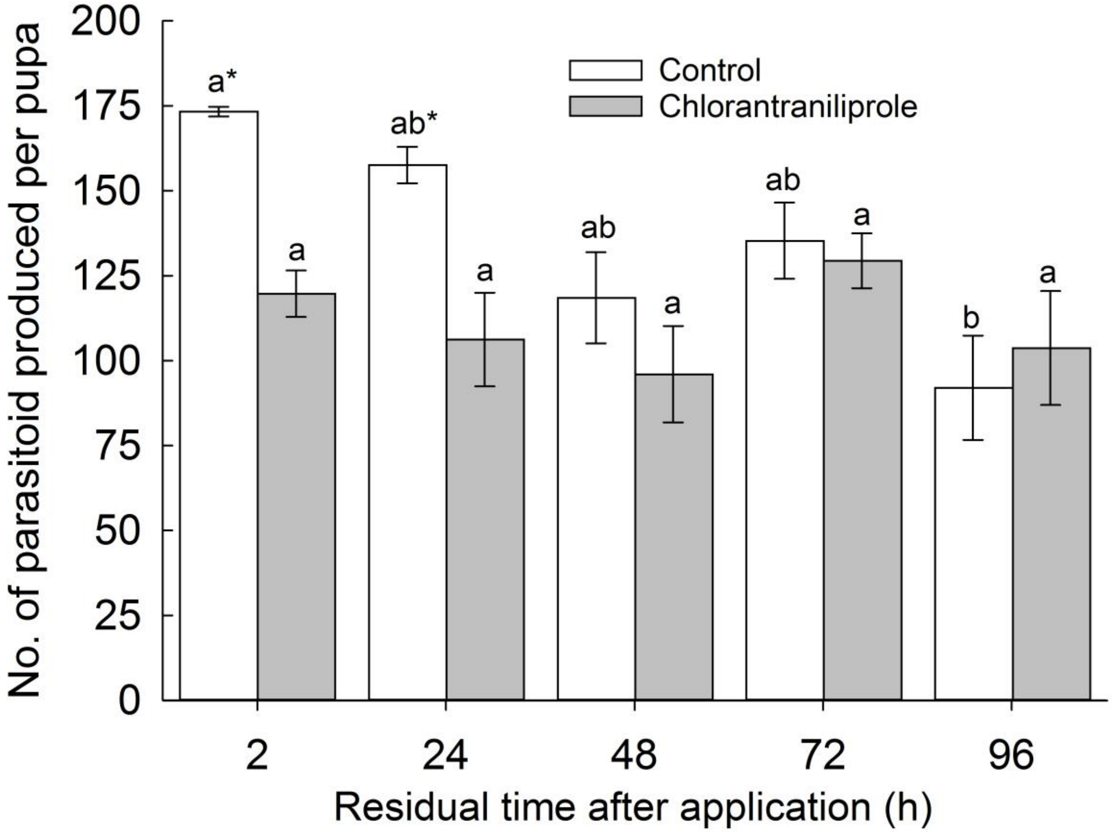

Figure 2. Number (mean + SE) of parasitoids produced from pupa of *Tenebrio molitor* parasitized by females of *Tetrastichus howardi* that survived contact with chlorantraniliprole and in the control group. Bars with different letters indicate statistical significance among residual intervals 2 to 96h after insecticide application (Tukey HSD, α = 0.01), and asterisks (*) indicate difference between chlorantraniliprole and control group in the same residual interval (Fisher's test, α = 0.05)

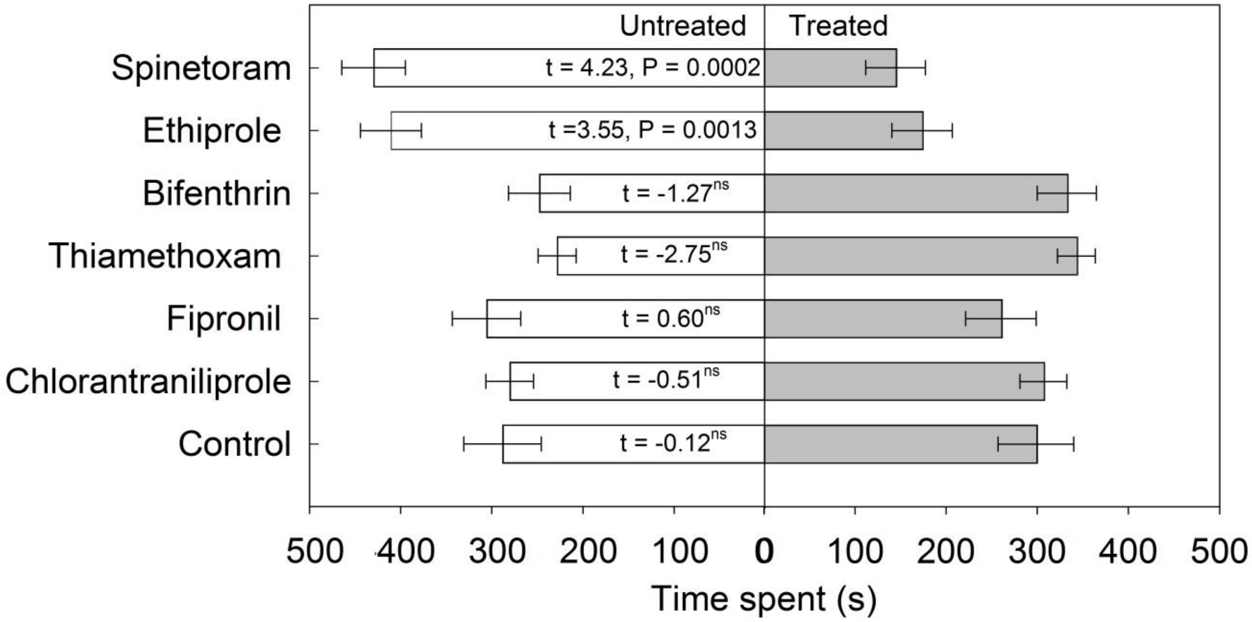

Figure 3. Time spent (mean ± SE) by *Tetrastichus howardi* females released in insecticide half-treated and half-untreated arenas and control arenas. Values inside bars means the statistical values of *t*-paired test comparing time between insecticide-treated and -untreated halves of the arena ($\alpha = 0.05$); while ^{ns} stands for values of *t* statistically non-significant.

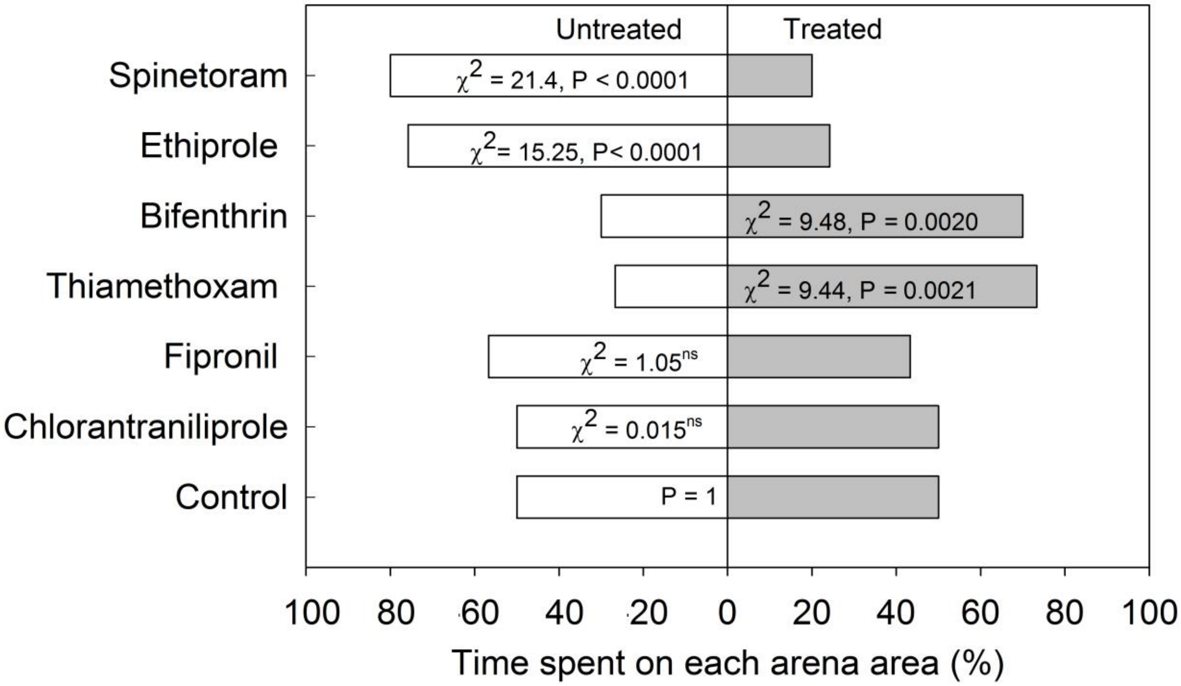

Figure 4. Percentage of time spent by *Tetrastichus howardi* females in insecticide-untreated and –treated halves areas of the arena. Values inside bars stand for pairwise comparisons between treated and untreated halves of the arena and in the control group by Wilcoxon's test (α = 0.05).

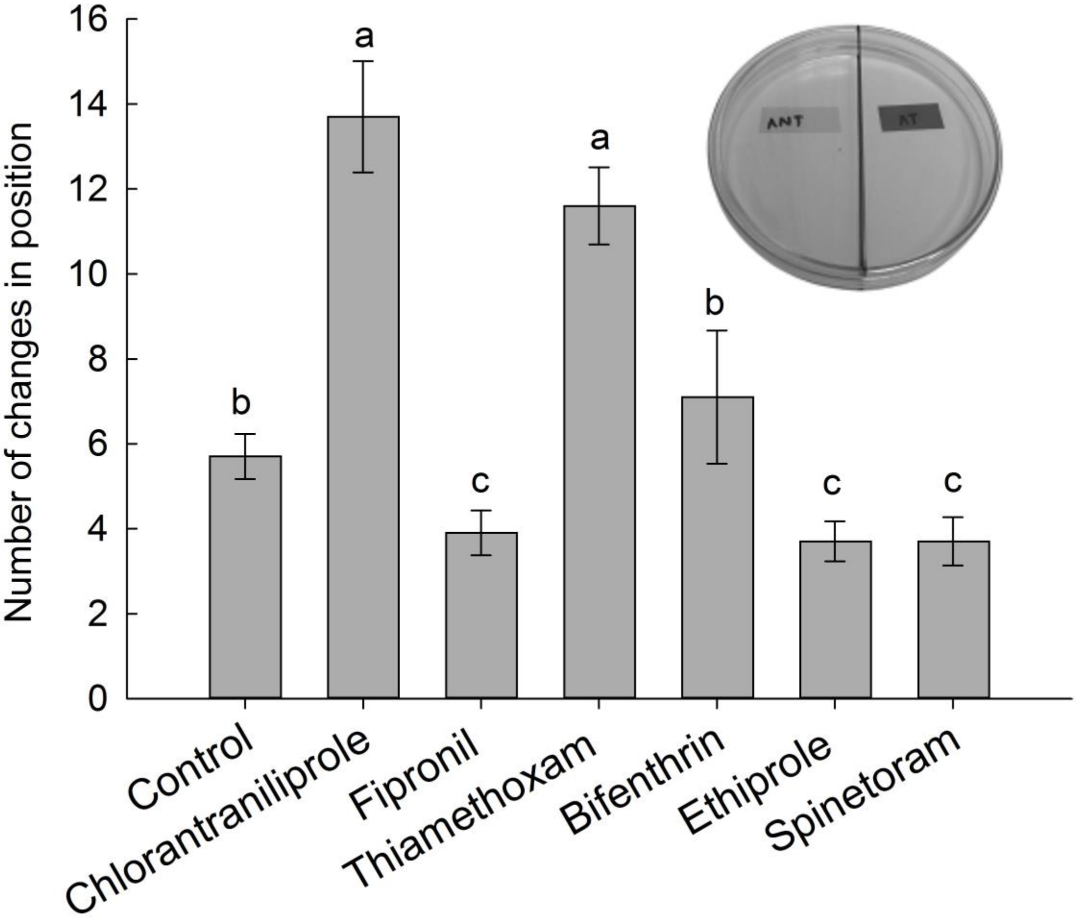

Figure 5. Number (mean ± SE) of change in position between insecticide half-treated (AT) and –untreated (ANT) areas of the arena by *Tetrastichus howardi* females and in the control arenas. Different letters indicate differences among treatments by Waller-Duncan K-ratio t-test ($\alpha = 0.007$)

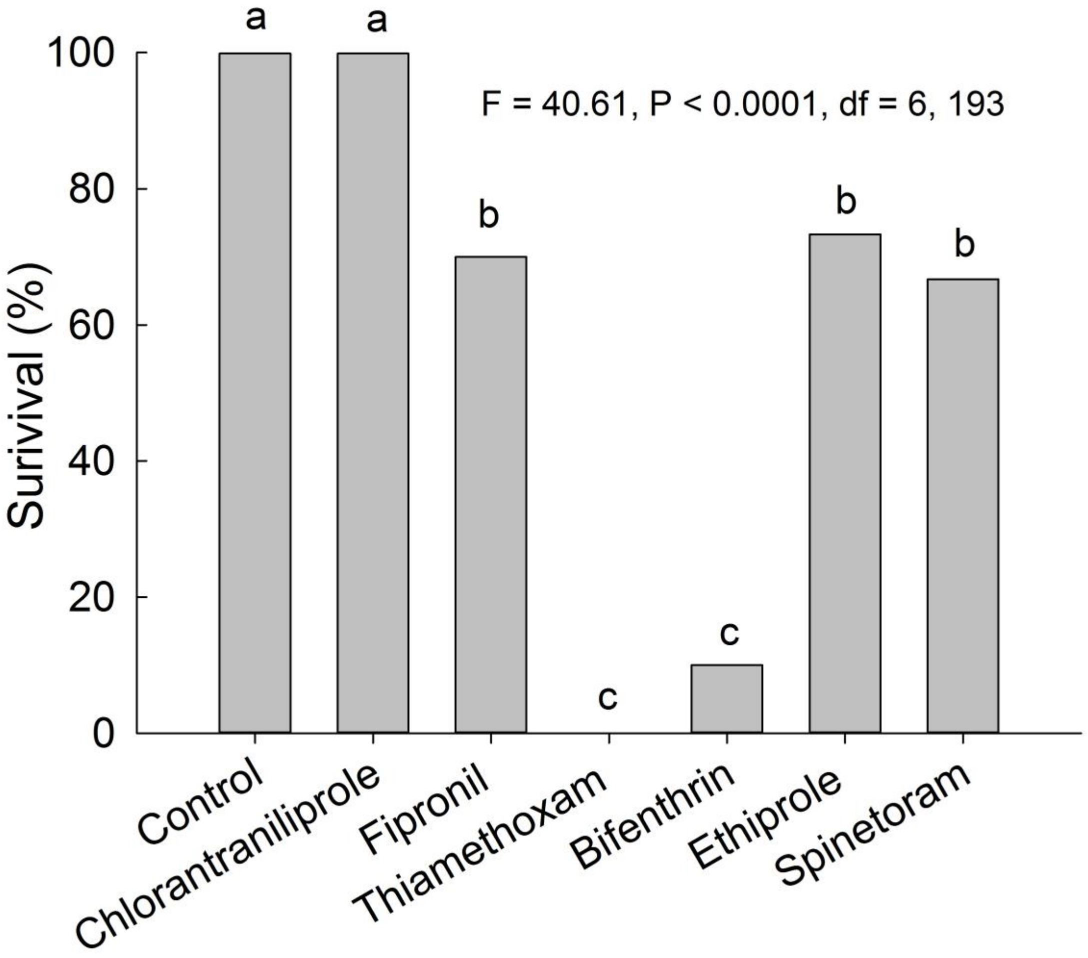

Figure 6. Female *Tetrastichus howardi* survival 24h after observation for 10 min in insecticide half-treated arenas and control arenas. Different letters indicate differences among treatments by Waller-Duncan K-ratio t-test (α = 0.007)

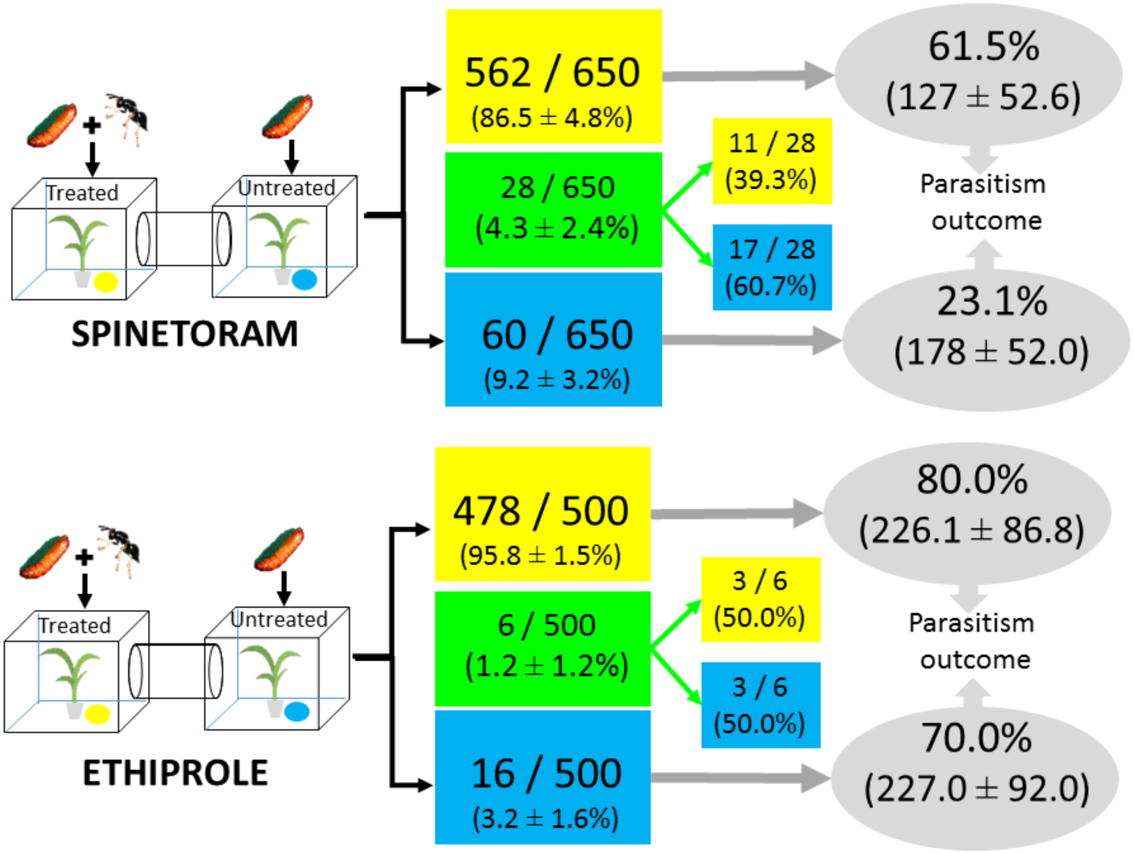

Figure 7. Number of *Tetrastichus howardi* females dead 24h after releasing 50 females inside cages containing one sugarcane plant treated with spinetoram or ethiprole paired with the cage containing one untreated plant, and final results for percentage of females surviving 24h after caging (treated cage = grey square; and untreated cage = dark grey square) and per treatment (smaller squares). Results in the gray circles represent the percentage of parasitism on sentinel pupae placed on the plants and the mean number of offspring produced per parasitized pupa. Mean values are followed by their respective 95% confidence intervals, when specified.

CAPÍTULO 4

CONSIDERAÇÕES FINAIS

No controle biológico aplicado (CBA), a produção de inimigos naturais em larga escala, e posterior liberação em campo, é fundamental para a implementação desta estratégia de controle de pragas. O uso de parasitoides no CBA, implica em criação continua usando hospedeiros, podendo ser empregado hospedeiros alternativos. A criação continua de parasitoides em hospedeiros alternativos podem resultar em perdas de qualidade biológica dos mesmos, bem como adaptação do parasitoide ao hospedeiro natal, o que pode ter efeitos diretos no CBA. Além disso, outro ponto relevante antes da liberação de parasitoides no campo é o conhecimento da interação com outras táticas de manejo, para evitar que estas possam interferir no comportamento e desempenho do parasitoide, diminuindo e até inviabilizando o controle almejado.

Os resultados apresentados neste estudo visam dar suporte para a criação em larga escala do parasitoide *Testrastichus howardi* (Olliff) (Hymenoptera: Eulophidae), referente ao uso de pupas de *Tenebrio molitor* L. (Coleoptera: Tenebrionidae). Este hospedeiro permitirá produção continua e a baixo custo do parasitoide. Os resultados mostram não haver perda de desempenho do parasitoide quando criado continuamente por até 30 gerações empregando este hospedeiro alternativo.

Fêmeas de *T. howardi* se dispersam após a localização e parasitismo de pupas de *D. saccharlis* no interior do colmo de cana. Contudo, a reposição de ovos em quantidade para um segundo parasitismo com sucesso não ocorre em intervalo de até 24 horas após o primeiro parasitismo. Os resultados indicam que a localização de outros hospedeiros neste curto período de tempo não necessariamente vai resultar em parasitismo com produção de descendentes, apesar de poder haver a mortalidade do hospedeiro atacado.

Em síntese:

- Fêmeas de *T. howardi* pode encontrar pupas da broca-do-colmo, parasitar, superparasitar e abandoná-las após o parasitismo;
- *Tetrastichus howardi* pode ser considerado uma espécie sinovigênica, com aproximadamente 50% dos ovos formados em 24 horas de idade, e aumentando a quantidade de ovos produzidos conforme envelhece;
- Fêmeas de *T. howardi* podem realizar múltiplas oviposições na mesma pupa de *D. saccharalis*, mas a primeira oviposição já é suficiente para matar a pupa, sem aumentar a produção de descentenes com as subsequentes oviposições;
- Fêmeas de *T. howardi* após a primeira oviposição reduz significativamente a quantidade de ovos no ovário, o qual não é recuperado em até 24 horas após o parasitismo;
- Nem todas as fêmeas de *T. howardi* após realizarem a primeira oviposição, realiza em sequência uma segunda oviposição, parasitando uma nova pupa de *D. saccharalis*;
- Fêmeas de *T. howardi* realizando uma segunda oviposição, em sequência, em outra pupa de *D. saccharalis* tem alta chance de matá-la, porém com redução significativa na viabilidade e quando viável, a produção de descendentes é significativamente inferior a progênie produzida pela primeira oviposição;
- Os inseticidas bifentrina, fipronil e tiametoxam, causaram alta e rápida mortalidade dos parasitoides, não sendo recomendadas para um manejo de pragas que pretende a preservação de inimigos naturais;

- Os inseticidas etiprole e espinetoram, apesar de serem tóxicos ao parasitoide, a mortalidade foi mais lenta, bem como o parasitoide foi capaz de parasitar pupas da praga e produzir descendentes em plantas contendo resíduo desses inseticidas;
- O inseticida clorantraniliprole foi compatível com o parasitoide *T. howardi* permitindo sua sobrevivência com capacidade de parasitar pupas e produzir descendência após exposição ao resíduo do inseticida.