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CAIROMÔNIOS DE PERCEVEJOS (HEMIPTERA: PENTATOMIDAE) ASSOCIADOS À APRENDIZAGEM DE PARASITOIDES DE OVOS (HYMENOPTERA: SCELIONIDAE)

Roberta Tognon
Bióloga (UCS) Mestre (UFRGS)

Tese apresentada como um dos requisitos
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ROBERTA TOGNON
Bióloga - UCS
Mestre em Fitotecnia - UFRGS

TESE

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Por

JOSUÉ SANT'ANA
Orientador - PPG Fitotecnia

SIMONE MUNDSTOCK JAHNKE
Coordenadora do Programa de
Pós-Graduação em Fitotecnia

SIMONE MUNDSTOCK JAHNKE
PPG Fitotecnia/UFRGS

RAÚL ALBERTO LAUMANN
EMBRAPA Recursos Genéticos e
Biotecnologia – Brasília, DF

DORI EDSON NAVA
EMBRAPA Clima Temperado
Pelotas/RS

CARLOS ALBERTO BISSANI
Diretor da Faculdade
de Agronomia

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CAIROMÔNIOS DE PERCEVEJOS (HEMIPTERA: PENTATOMIDAE) ASSOCIADOS À APRENDIZAGEM DE PARASITOIDES DE OVOS (HYMENOPTERA: SCELIONIDAE)[1]

Autor: Roberta Tognon
Orientador: Dr. Josué Sant'Ana

RESUMO

Parasitoides Scelionidae são agentes de controle natural de percevejos e seu sucesso de parasitismo está vinculado a estratégias de busca (inatas e/ou aprendidas), sendo os cairomônios provenientes de ovos hospedeiros, uma das principais pistas utilizadas por estes organismos. Portanto, este trabalho objetivou verificar a influência do hospedeiro de origem em sucessivas gerações dos scelionídeos *Telenomus podisi* Ashmead e *Trissolcus basalis* (Wollaston) através de testes de escolha a ovos dos pentatomídeos *Euschistus heros* (Fabricius), *Piezodorus guildinii* (Westwood) e *Nezara viridula* (L.). Também foram identificados compostos químicos presentes em ovos de *E. heros*, *Euschistus conspersus* Uhler e *Halyomorpha halys* Stål. O efeito cairomonal e de aprendizagem de extratos e misturas sintéticas destes em *T. podisi* e *Trissolcus erugatus* Johnson em laboratório, semicampo e/ou campo foram subsequentemente observados. Verificou-se que o parasitismo de *T. podisi* é influenciado pelo hospedeiro de origem, no entanto, *T. basalis* apresentou uma preferência por ovos de *N. viridula*, independente do hospedeiro de criação. Observamos que o extrato de ovos de *E. heros* é atrativo à *T. podisi* e deste foram identificados 32 compostos, sendo que a mistura de quatro destes (limoneno, canfeno, benzaldeído e β-pineno) foi responsável pela atratividade e incremento do parasitismo de *T. podisi* em ovos de *E. heros*, tanto em laboratório como semicampo. Quanto à *E. conspersus*, a atratividade de *T. podisi* e *T. erugatus* ao extrato de ovos foi confirmada em bioensaios quimiotáxicos, sendo o metil (2*E*,4*Z*)-2,4-decadienoato, a principal substância elucidada. O extrato de ovos e as substâncias sintéticas provenientes da identificação química (hexadecanal, octadecanal e eicosanal) de ovos de *H. halys* repeliram *T. podisi* e *T. erugatus*, inviabilizando o parasitismo. Além disso, a experiência em substâncias cairomonais de ovos fez com que as vespas alterassem seu comportamento inato, independente de qual era seu hospedeiro de origem. Essa aprendizagem pôde, por vezes, persistir ao longo de alguns dias, tornando os insetos mais aptos na busca e parasitismo. Os resultados deste trabalho indicam que é possível manipular o comportamento de parasitoids utilizando semioquímicos. O conhecimento sobre a aprendizagem dos insetos pode auxiliar em estratégias de liberação e manutenção destes organismos para o incremento do controle biológico em agroecossistemas.

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STINK BUG KAIROMONES (HEMIPTERA: PENTATOMIDAE) ASSOCIATED TO LEARNING OF EGG PARASITOIDS (HYMENOPTERA: SCELIONIDAE)[1]

Author: Roberta Tognon
Adviser: Dr. Josué Sant'Ana

ABSTRACT

Scelionidae parasitoids are important natural enemies of stink bugs and their parasitism success is related to innate and/or learned strategies used for host location, in which egg kairomones play an important role. Thus, the objective of this study was to verify the influence of original host over generations of *Telenomus podisi* Ashmead and *Trissolcus basalis* (Wollaston) through a parasitism choice test in *Euschistus heros* (Fabricius), *Piezodorus guildinii* (Westwood) and *Nezara viridula* (L.) egg masses. Moreover, chemical identification of substances present on the external layer of *E. heros*, *Euschistus conspersus* Uhler and *Halyomorpha halys* Stål eggs was also done. Their kairomonal activity and learning behavior to extracts and synthetic mixtures were evaluated in *T. podisi* and *Trissolcus erugatus* under laboratory, semi-field and/or field conditions. We observed that parasitism of *T. podisi* is influenced by its original host, however, *T. basalis* showed a preference for parasitize *N. viridula* eggs, independently of the host rearing. Furthermore, *T. podisi* is attracted by *E. heros* egg extract, which we identified 32 compounds and a mixture of four of them (limonene, camphene, benzaldehyde and β-pinene) induced attractiveness and increased parasitism by *T. podisi* in *E. heros* egg masses. Regarding to *E. conspersus*, attraction of *T. podisi* and *T. erugatus* to egg extract was registered in chemotaxic assays, being methyl (2*E*,4*Z*)-2,4-decadienoate the main substance found. Egg extract and the synthetic blend from *H. halys* eggs (hexadecanal, octadecanal and eicosanal) repelled either *T. podisi* or *T. erugatus*, resulting in parasitism absence. Besides, experienced females on kairomonal substances from eggs of a specific host, changed their innate behavior and, sometimes, it was kept for days, becoming wasps ablest to search and parasitize. Thus, these results showed that it is possible to manipulate parasitoid behavior using semiochemicals. A better understanding of parasitoids learning behavior can help establishing strategies to release and/or maintenance these organisms in agroecosystems for biological control improvement.

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1 INTRODUÇÃO

Parasitoides de ovos de Scelionidae são agentes de controle natural de percevejos fitófagos (Pentatomidae) em agroecossistemas, os quais são pragas de plantas cultivadas, alimentando-se principalmente de sementes e frutos imaturos.

A utilização desses parasitoides é uma das técnicas que vem assumindo grande importância em Programas de Manejo Integrado de Pragas (MIP), principalmente em um momento de ascensão da produção integrada e da orgânica, objetivando uma agricultura mais sustentável.

O sucesso do controle biológico aplicado e/ou conservativo de insetos está diretamente relacionado a um estudo mais aprofundado dos fatores que interferem nas interações entre hospedeiro e parasitoide. Dentre estes, fazem parte os que intermediam a comunicação química entre inimigos naturais e herbívoros, assim como a influência do hospedeiro de origem e da aprendizagem e memória no comportamento de busca e reconhecimento de espécie (s) alvo e, possivelmente, no sucesso de parasitismo.

Sendo assim, este trabalho teve como objetivo conhecer os aspectos que intermedeiam as relações tróficas entre parasitoides de ovos e pentatomídeos, enfatizando os seguintes aspectos: a) impacto do hospedeiro de origem na escolha de *Telenomus podisi* Ashmead (Hymenoptera: Scelionidae) e *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) em diferentes hospedeiros; b) identificação química de substâncias voláteis provenientes da camada externa de ovos de pentatomídeos; c) quimiotaxia e parasitismo de *T. podisi* e *Trissolcus erugatus* Johnson (Hymenoptera: Scelionidae) frente a extratos e compostos sintéticos de ovos, bem como sua capacidade de aprendizagem e memória em relação a estas substâncias; f) efeito cairomonal de compostos identificados em ovos e parasitismo em condição de laboratório, semicampo e/ou campo.

Os resultados estão apresentados na forma de cinco artigos. O primeiro deles intitulado “Is it possible to manipulate egg parasitoids’ preference for a target host?” formatado para a submissão em “BioControl”. Os demais artigos, intitulam-se: “Kairomones from *Euschistus heros* egg masses and their potential use for *Telenomus podisi* parasitism improvement” formatado de acordo com as normas do periódico “Entomologia Experimentalis et Applicata”. O terceiro, “Volatiles mediating parasitism of *Euschistus conspersus* and *Halyomorpha halys* eggs by *Telenomus podisi* and *Trissolcus erugatus*” encontra-se publicado no periódico “Journal of Chemical Ecology”. “Are *Halyomorpha halys* (Hemiptera: Pentatomidae) eggs protected from endemic parasitoids by chemical defenses?” está formatado de acordo com o periódico “Biological Control” e como último artigo desta tese, “Hopkins’ host selection principle revisited: learning and memory by *Telenomus podisi* and *Trissolcus erugatus* (Hymenoptera: Scelionidae) to parasitize *Halyomorpha halys* (Hemiptera: Pentatomidae) eggs” está formatado seguindo as normas do periódico “Environmental Entomology”.

2 REVISÃO BIBLIOGRÁFICA

2.1 Pentatomídeos de importância agrícola: aspectos bioecológicos, danos e controle

Pentatomidae é a quarta família mais números Heteroptera, incluindo mais de 4.700 espécies em mais de 800 gêneros (Grazia *et al*., 2015). Dentro dessa, existem oito subfamílias: Asopinae, Cyrtocorinae, Discocephalinae, Edessinae, Pentatominae, Phyllocephalinae, Podopinae e Serbaninae, sendo Pentatominae a maior do grupo (Schuh & Slater, 1995). Danos ocasionados em sementes/cereais, leguminosas e frutos de plantas cultivadas, fazem algumas das espécies desta subfamília destacarem-se como economicamente importantes (Panizzi *et al*., 2000). No Brasil a principal cultura que sofre perda considerável com esses insetos é a da soja [*Glycine max* L. (Fabales: Fabaceae)] (Hoffmann-Campo *et al*., 2000), porém o arroz [*Oryza sativa* L. (Poales: Poaceae)] (Martins *et al*., 2004), o trigo (*Triticum* spp.) (Panizzi *et al*., 2016) e o algodão [(*Gossypium hirsutum* L.) (Malvales: Malvaceae)] (Soria *et al*., 2016), também são afetados. Nos Estados Unidos da América (EUA) espécies deste grupo também são importantes pragas nas culturas do tomateiro [*Solanum lycopersicum* L. (Solanales: Solanaceae)] (Cullen & Zalom, 2005), da soja (Nielsen *et al*., 2011), milho [*Zea mays* L. (Poales: Poaceae)] (Cissel *et al*., 2015), pêssego [*Prunus persica* L. (Rosales: Rosaceae)] maçã [*Malus* spp. (Rosales: Rosaceae)] (Joseph *et al*., 2015) entre outras.

Dentre o complexo de pentatomídeos encontrado na cultura da soja no Brasil, os mais frequentes são: *Euschistus heros* (Fabricius), *Nezara viridula* (L.) e *Piezodorus guildinii* (Westwood) (Panizzi & Slansky Jr., 1985a; Hoffmann-Campo *et al*., 2000; Panizzi *et al*., 2000). Nos EUA outras espécies, como *Euschistus conspersus* Uhler (Cullen & Zalom, 2005) e *Halyomorpha halys* Stål (Joseph *et al*., 2015) são pragas-chave na produção de frutos, cereais e leguminosas.

2.1.1 *Euschistus heros*

O percevejo-marrom é nativo da Região Neotropical e no Brasil está presente em abundância nas regiões mais quentes como o norte do Paraná e no Centro-Oeste, o que favorece um maior número de gerações dessa espécie (Panizzi & Slansky Jr., 1985a; Panizzi, 1997; Azambuja *et al*. 2013).

As posturas são constituídas de cinco a oito ovos de cor amarela, depositados principalmente nas folhas ou nas vagens da soja (Hoffmann-Campo *et al*., 2000). Os adultos são de coloração marrom escuro, apresentam espinhos no pronoto e atingem, em média, 12,08 mm de comprimento (Panizzi & Niva, 1994). Costa *et al*. (1998) observaram que a fase de ovo de *E. heros* tem duração aproximada de sete dias, sendo que os tempos de desenvolvimento do primeiro ao quinto ínstares foram de 4,6 ± 0,9; 7,1 ± 0,6; 5,7 ± 1,5; 7,0 ± 2,2 e 7,1 ± 1,3 dias, respectivamente. Fêmeas e machos possuem uma longevidade média de 50 dias atingindo a maturidade sexual por volta de 11, com fecundidade aproximada de 130 ovos/fêmea (24 ± 0,5 °C; 70 ± 10% UR; fotofase de 14 horas). Na época de entressafra, alimentam-se de plantas alternativas como *Amaranthus retroflexus* L. (Caryophyllales: Amaranthaceae) e *Vassobia breviflora* (Sendtn.) (Solonales: Solanaceae) (Medeiros & Megier, 2009) e nas regiões mais frias, deslocam-se para abrigos de sobrevivência, alojando-se sob folhas secas de plantas como *Mangifera indica* L. (Sapindales: Anacardiaceae) (Panizzi & Niva, 1994; Godoy *et al*., 2010), *Cajanus cajan* L. (Fabales: Fabaceae), *Helianthus annus* L. (Asterales: Asteraceae), *Acanthospermun hispidium* L. (Asterales: Asteraceae) e *Pterogyne nitens* Tul. (Fabales: Fabaceae) (Panizzi & Niva, 1994; Mourão & Panizzi, 2000) permanecendo em estado de diapausa até a próxima safra de verão.

2.1.2 *Nezara viridula*

O percevejo-verde tem uma distribuição cosmopolita, habita as regiões Tropical, Subtropical e Temperadas da Europa, Ásia, África e das Américas (Lethierry & Severian, 1893; Todd, 1989; Panizzi *et al*., 2000). É provável que seu centro de origem seja a Etiópia, no continente Africano (Jones, 1988; Todd, 1989). A duração média da fase imatura (25 ± 28 ºC, 55 ± 65% UR, 14h fotofase), foi de 42,4 ± 1,4 dias, sendo a de ovo de 4,9 ± 1,7 dias e a dos ínstares, do primeiro ao quinto, respectivamente, de 4,4 ± 0,1; 5,7 ± 0,5; 5,6 ± 0,1; 9,6 ± 0,8 e 12,3 ± 0,9 dias (Harris & Todd, 1980). A maturidade sexual, segundo Mitchell & Mau (1969), é atingida entre 5 a 17 dias, dependendo das condições do ambiente.

O adulto é de coloração totalmente verde, com tamanho entre 12 e 15 mm, podendo sobreviver até 70 dias. Os ovos são de coloração amarelo claro e, normalmente, são depositados na face inferior das folhas, em massas regulares de 50-100 ovos (Hoffmann-Campo *et al*., 2000). Essa espécie é polífaga e no norte do Paraná, completa duas ou três gerações em soja, de dezembro a abril, podendo ser também comumente encontrada em feijão (*Phaseolus vulgaris* L. [Fabales: Fabaceaea]). Posteriormente, utiliza várias plantas hospedeiras como *Acanthospermum hispidum* D.C. (Asterales: Asteraceae), *Ricinus communis* L. (Malpighiales: Euphorbiaceae), *Desmodium tortuosum* (Swartz) D.C.

(Fabales: Fabaceae), *Crotalaria* sp. (Fabales: Fabaceae), *Tritucum aestivum* L. (Poales: Poaceae), *Leonurus sibiricus* L. (Lamiales: Lamiaceae) (Panizzi, 1997; Hoffmann-Campo *et al*., 2000).

No período de entressafra, no sul do Paraná, em Santa Catarina e no Rio Grande do Sul, o percevejo-verde se abriga principalmente sob cascas de árvores (Hoffmann-Campo *et al*., 2000). Neste período a espécie entra em diapausa, trocando de cor, passando de verde para castanho arroxeado (Musolin *et al*., 2007; Musolin, 2012).

2.1.3 *Piezodorus guildinii*

O percevejo-verde-pequeno é um pentatomídeo Neotropical com ampla distribuição geográfica, sendo encontrado do sul dos EUA até a Argentina (Panizzi & Slansky Jr., 1985a). No Brasil, ocorre desde a região tradicional de cultivo da soja (Sul e Centro-Oeste) até as regiões Norte e Nordeste do país (Hoffmann-Campo *et al*., 2000).

O adulto tem, aproximadamente, 10 mm de comprimento e cor verde amarelada e apresenta uma listra transversal marrom avermelhada no pronoto. Os ovos são pretos, colocados em fileiras pareadas, em número de 10 a 20 por postura e, preferencialmente, são depositados nos legumes. As ninfas recém-eclodidas medem cerca de 1 mm, possuem comportamento gregário, permanecendo próximas à postura (Hoffmann-Campo *et al*., 2000).

Esta espécie também é encontrada em feijão, ervilha [*Pisum sativum* L. (Fabales: Fabaceae)], alfafa [*Medicago sativa* L. (Fabales: Fabaceae)], girassol e algodão [*Gossypium* spp. (Malvales: Malvaceae), porém não é considerada uma praga nestas culturas (Panizzi & Slansky Jr., 1985b). Entre as plantas nativas, alimenta-se de legumes de plantas dos gêneros *Sesbania* e *Crotalaria* (Panizzi, 1987; Panizzi & Slanksy Jr., 1985b).

2.1.4 *Euschistus conspersus*

Euschistus conspersus é uma espécie endêmica da América do Norte (Beers et al. 1993), sendo considerada uma praga chave na produção de maças (Ehler, 2000) e tomates (Cullen & Zalom, 2005). Apresenta a superfície ventral do corpo amarelo-esverdeada e a dorsal marrom-acinzentada (Hunter & Leight, 1965).

A incubação de ovos e o desenvolvimento das ninfas até a fase adulta melhor ocorrem entre 27 e 32°C. A duração média dos estágios de vida é de 6,2 dias para a fase de ovo e de 25,6 dias para as fases de ninfa. Os machos podem viver cerca de 128,2 e fêmeas 75,1 dias (Hunter & Leight, 1965). Fêmeas ovipositam entre o 10° e 32° dia de vida, com uma média total de 225 ovos. Durante o inverno, os adultos entram em diapausa e permanecem sob folhas de amoreira [*Rubbus* sp. (Rosales: Rosaceae)] (Ehler 2000).

2.1.5 *Halyomorpha halys*

Espécie nativa da Ásia (Hsiao, 1977), sendo neste continente uma praga de leguminosas, sementes, frutos e plantas ornamentais (Kobayashi *et al.*, 1972, Funayama 1996, Choi *et al.*, 2000, Toyama *et al*., 2006). Desde que se estabeleceu nos EUA, *H. halys* tem causado perdas em muitas culturas, dentre elas destacam-se a soja (Nielsen *et al*., 2011), milho (Cissel *et al*., 2015), pêssego e maçã (Joseph *et al*., 2015), pêra [*Pyrus* spp. (Rosales: Rosaceae)] e frutas cítricas (Hoebecke & Cartner, 2003), tomate, berinjela [*Solanun melongena* L. (Solanales: Solanaceae)] (Kuhar *et al*., 2012), em cultivos de pequenas frutas vermelhas, como o mirtilo [*Vaccinium myrtillus* L. (Ericales: Ericaceae)] (Wiman *et al*., 2015) entre outros.

As ninfas eclodem entre 6-7 dias após a deposição dos ovos e os cinco estágios ninfais duram aproximadamente 43 dias. Em média, uma fêmea oviposita 168 ovos entre o 14° ao 25° dia de vida, com um período de 18 dias de oviposição. Machos são menores que as fêmeas apresentando um tamanho de 12 e 14,4 mm, respectivamente (Medal *et al.*, 2013).

A população hibernante do inverno se abriga invadindo residências e galpões rurais (Hamilton *et al.*, 2008, Nielsen *et al.*, 2008). Devido ao comércio entre o Sul da América Latina e os EUA existe uma grande possibilidade dessa espécie se estabelecer nos países latino-americanos como Chile, Argentina, Uruguai e os estados do Sul do Brasil, pois as condições climáticas e os hospedeiros presentes nestas regiões favorecem seu desenvolvimento (Zhu *et al.*, 2012).

2.2 Controle biológico: parasitoides

A principal forma de controle de percevejos é o químico (Panizzi *et al.*, 2000). Os produtos mais utilizados são inseticidas dos seguintes grupos químicos: neonicotinoides, piretroides e organofosforados (Nielsen *et al.*, 2008; Natwick *et al.*, 2015; AGROFIT, 2016). No entanto, os efeitos indesejáveis desse método podem ser evitados ou minimizados com a adoção de técnicas que fazem parte do Manejo Integrado de Pragas (MIP). Neste contexto o controle biológico é uma das técnicas que vem assumindo grande importância em programas de manejo, principalmente a partir de um momento em que se passou a discutir questões relacionadas à produção integrada e orgânica rumo a uma agricultura sustentável, voltada à qualidade do ambiente e da saúde pública (Parra *et al.*, 2002).

O controle biológico é um fenômeno natural, que regula o número de plantas e animais pelos inimigos naturais e foi definido por DeBach (1968) como a ação de parasitoides, predadores e patógenos na manutenção da densidade de outro organismo a um nível mais baixo do que aquele que normalmente ocorreria nas suas ausências.

Parasitoides são insetos que se desenvolvem em um hospedeiro, alimentando-se do mesmo e, obrigatoriamente, causando sua morte (Vinson, 1976). O parasitismo pode ocorrer em diferentes fases de desenvolvimento do hospedeiro, deste modo, os insetos são classificados em parasitoides de ovo, larva, pupa ou do estágio adulto (Costa *et al*., 2006).

Os microhimenópteros pertencentes à Scelionidae (Talamas & Buffington, 2015), são agentes de controle natural de percevejos na cultura do arroz (Martins *et al*., 2004), soja (Medeiros *et al*., 1997; Pacheco & Corrêa-Ferreira, 2000), milho (Tillman, 2010), algodão, amendoim (Tillman, 2011), entre outras. As principais espécies de parasitoides de ovos registradas em *E. heros*, *E. conspersus*; *N. viridula P. guildinii e H. halys* (Hemiptera: Pentatomidae) pertencem aos gêneros *Telenomus* e *Trissolcus* (Medeiros *et al*., 1997; Pacheco & Corrêa-Ferreira 2000; Godoy *et al*., 2005; Maciel *et al*., 2007; Haye *et al*., 2015; Pease & Zalom 2010).

Para *E. heros*, na cultura da soja, Pacheco & Corrêa-Ferreira (2000) observaram, no estado do Paraná, índice superior a 80%, por *T. podisi* durante o período de enchimento de grãos até a maturação, alcançando 100% de ovos parasitados no final do ciclo da cultura. Estudo semelhante foi conduzido por Godoy *et al*. (2005), os quais observaram 62,5% das posturas de *E. heros* parasitadas e 23,8% das de *P. guildinii* coletadas no estado do Mato Grosso do Sul.

2.2.1 Aspectos bioecológicos de Scelionidae

Os scelionídeos são pequenos himenópteros (na maioria, inferiores a 2,5 mm de comprimento) encontrados em todos os habitats, com exceção das regiões polares, sendo estes diversos e abundantes em florestas úmidas, tropicais e subtropicais (Austin *et al*., 2005). Esta família possui 4.500 espécies descritas (Masner & Hanson, 2006; Murphy *et al*., 2007) as quais, na maioria, são endoparasitoides de ovos de aranhas e de insetos dos grupos Hemiptera, Odonata, Orthoptera, Mantodea, Neuroptera, Coleoptera, Diptera e Lepidoptera (Austin *et al*., 2005; Masner & Hanson, 2006). Muitas espécies apresentam um alto grau de especificidade hospedeira, restringindo-se, normalmente, a apenas uma família de artrópode (Masner & Hanson, 2006).

No contexto agrícola, a principal subfamília de Scelionidae é Telenominae, devido ao fato de representantes deste grupo serem relatados como agentes de controle biológico de Heteroptera e Lepidoptera. A maioria dos insetos deste grupo evita o superparasitismo, pois as fêmeas detectam ovos já parasitados através de marcadores externos (feromônio de oviposição) (Masner & Hanson, 2006).

Dentre os telenomíneos, *T. basalis* e *Telenomus remus* Nixon, já foram introduzidos em alguns países para combater, respectivamente, *N. viridula* (Hoffmann *et al*., 1991; Corrêa-Ferreira & Moscardi, 1996) e *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae) (Ferrer, 1998). *Telenomus podisi* também apresenta um elevado desempenho como regulador populacional em agroecossistemas nas culturas da soja, milho, algodão e amendoim [*Arachis hypogaea* L. (Fabales: Fabaceae)] (Pacheco & Corrêa-Ferreira, 2000; Ehler 2000; Tillman, 2010, 2011).

2.3 Comunicação química de parasitoides de ovos

As substâncias químicas envolvidas na comunicação entre os organismos são denominadas semioquímicos, definidos como qualquer substância que, quando liberada por um determinado organismo no ambiente, provoque uma mudança fisiológica e/ou comportamental em outro. Nos insetos, estes são os principais responsáveis pelo comportamento reprodutivo, localização e seleção do hospedeiro, do habitat e, no caso de insetos sociais, da organização da colônia. Estas substâncias podem ter ação intraespecífica (feromônio) ou interespecífica (aleloquímico) (Nordlund & Lewis, 1976).

As estratégias de busca e o sucesso do parasitismo envolvem uma série de etapas (localização do habitat e do hospedeiro, reconhecimento, aceite e oviposição) e dependem de estímulos que podem ser de natureza química, física e/ou morfológica (Vinson, 1984; Vet *et al*., 1995; Afsheen *et al*., 2008; Conti & Colazza, 2012). Parasitoides utilizam, principalmente, semioquímicos na localização hospedeira e, dessa forma, desenvolveram estratégias as quais possibilitaram maior eficiência de encontros e, consequentemente, maior viabilidade de sobrevivência da prole (Vinson, 1985; 1998; Vet & Dicke, 1992; Steidle & van Loon, 2002; Fatouros *et al*., 2008).

Diversas espécies de parasitoides de ovos utilizam feromônios de hospedeiros como cairomônios e associam essas pistas com áreas onde as posturas poderão ser encontradas (Fatouros *et al*., 2008; Colazza *et al*., 2010). Os cairomônios, por sua vez, são sinais que quando liberados, beneficiam somente o agente receptor, sendo utilizados por parasitoides em estratégias de busca (Vilella & Della Lúcia, 2001; Corrêa & Sant'Ana, 2007).

As antenas têm um fundamental papel na detecção de vários semioquímicos, tanto na percepção de substâncias voláteis dispersas no ar e/ou por contato (compostos pouco voláteis). Em vespas Platygastroidea, os segmentos apicais das antenas de fêmeas formam uma clava compreendendo 3-7 segmentos, no lado inferior de cada um destes, entre as numerosas sensilas táteis, estão as denominadas "olfativas multiporosas" as quais são responsáveis pelo reconhecimento de sinais associados a hospedeiros através de proteínas receptoras, denominadas de *Odor Binding Proteins* (*OBPs*), que agem no transporte da molécula de estímulo até a membrana dendrítica (Bin, 1981; Isidoro *et al*., 1996; 2001).

Substâncias feromonais tem sido intensivamente estudadas na interação Heteroptera/Scelionidae em trabalhos de campo (Borges *et al*., 1998a; Bruni *et al*., 2000) e laboratório (Mattiaci *et al*., 1993; Borges *et al*., 1999; Colazza *et al*., 1999; Borges *et al*., 2003; Silva *et al*., 2006; Laumann *et al*., 2009). Essa interação cairomonal envolvendo pentatomídeos foi proposta por Silva *et al.* (2006), os quais observaram que *T. podisi* desencadeia resposta motora positiva frente a um dos compostos do feromônio sexual proveniente de machos de *E. heros*. Laumann *et al.* (2009) também constataram comportamento semelhante em *T. basalis* e *T. podisi*, para as substâncias sintéticas de defesa (4-oxo- (*E*)-2-hexanal e tridecano) presentes em glândulas metatorácicas de *N. viridula* e *E. heros*. Além do mais, substâncias presentes nas posturas e no córion de ovos de Pentatomidae, também podem atuar como cairomônios a curtas distâncias (Bin *et al*., 1993; Vet *et al*., 1995).

2.3.1 Voláteis cairomonais relacionados a ovos de hospedeiros

Espécies de famílias especializadas de parasitoides de ovos como Trichogrammatidae, Scelionidae e Mymaridae, além de Eulophidae e Encyrtidae, utilizam pistas químicas provenientes de ovos de hospedeiros como um cairomônio de localização e/ou reconhecimento. A família que mais tem sido estudada é Trichogrammatidae, seguida de Scelionidae, em ovos de Lepidoptera e Heteroptera, respectivamente (Colazza *et al*., 2010).

A atividade cairomonal de ovos de Pentatomida a parasitoides está associada ao material adesivo presente nestes, o qual fixa uns aos outros e os mesmos ao substrato (Bin *et al*., 1993; Borges *et al*., 1999). Segundo Bin *et al*. (1993), o cairomônio liberado pela substância encontrado nas posturas de *N. viridula* tem um importante papel no reconhecimento de ovos por *T. basalis* a curtas distâncias e o mesmo, pode ser facilmente removido por solventes químicos.

Bin *et al*. (1993) observaram que *T. basalis* utiliza principalmente pistas químicas na avaliação do hospedeiro, porém cor, espessura do córion, tamanho e a forma dos ovos também podem ser importantes indicativos de qualidade utilizados por estes insetos. Em ovos de heterópteros, a substância cairomonal está presente na secreção adesiva proveniente de células foliculares dos ovaríolos de fêmeas hospedeiras (Bin *et al*., 1993; Borges *et al*., 1999; Conti *et al*., 2003), sendo constituída de proteínas e mucopolissacarídeos (Bin *et al*., 1993), porém recentemente, algumas espécies de pentatomídeos tiveram os compostos elucidados e seu efeito cairomonal observado em scelionídeos (Michereff *et al.* 2016; Tognon *et al*. 2016). De acordo com Borges *et al*. (1999), fêmeas de *T. podisi* localizam ovos de *E. heros* e são capazes de distinguir posturas férteis de inférteis deste pentatomídeo, o que provavelmente deve-se ao fato de as inférteis possuírem uma quantidade menor de substâncias cairomonais.

Entre os Trichogrammatidae, *Trichogramma brassicae* Bezdenko (Renou *et al*., 1989; 1992; Frenoy *et al*., 1992) e *Trichogramma ostriniae* Pang & Chen (Bai *et al*., 2004; Yong *et al*., 2007) são as espécies mais investigadas. De acordo com Renou *et al*. (1992), fêmeas de *T. brassicae* respondem em olfatômetro para voláteis de ovos de *Ostrinia* *nubilalis* Hübner (Lepidoptera: Crambidae) e para extratos oriundos de ovos de *O. nubilalis* e *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae). Segundo os autores, as maiores respostas quimiotáxicas observadas foram para a mistura de cinco hidrocarbonetos saturados sintéticos presentes nos extratos, em comparação com os mesmos testados isoladamente. Assim como constatado em *T. brassicae*, fêmeas de *T. ostriniae* também respondem a extratos e massas de ovos do hospedeiro, neste caso, *Ostrinia furcanalis* (Guenée) (Lepidoptera: Crambidae), além disso, as mesmas tem a capacidade de desencadear respostas quimiotáxicas ao extrato de glândulas acessórias de fêmeas copuladas deste lepidóptero (Bai *et al*., 2004; Yong *et al*., 2007).

Na interação Pentatomidae/Scelionidae, Conti *et al*. (2003) constataram a atratividade a ovos do hospedeiro *Murgantia histrionica* (Hahn) (Hemiptera: Pentatomidae) em relação a *Trissolcus brochymenae* (Ashmead) (Hymenoptera: Scelionidae). Em olfatômetro tipo "Y", fêmeas do parasitoide foram atraídas aos voláteis de massas de ovos deste percevejo. Neste estudo, os autores também verificaram em uma arena aberta, a orientação de fêmeas em direção a massas de ovos e esferas de vidro tratadas com extratos de ovos de *M. histrionica*.

2.4 Seleção hospedeira, aprendizagem e memória de parasitoides de ovos

A busca do parasitoide por hospedeiros inicia com um comportamento inato ou instintivo (Papaj & Lewis, 1993). Este é guiado por padrões de ação fixos (*FAPs*), nos quais atuam mecanismos neurossensoriais, que desencadeiam uma sequência pré-programada de comportamentos (reação em cadeia) dependentes da presença de estímulos externos (Mathews & Mathews, 2010). No entanto, pode ser modificado através do contato sucessivo com determinado hospedeiro (experiência), podendo resultar em uma aprendizagem (Vet *et al*., 1995; Vinson, 1998).

Segundo Alcock (2005) e Matthews & Matthews (2010), a aprendizagem é definida como uma mudança permanente ou com durabilidade longa do comportamento, sendo considerada uma adaptação para lidar com as imprevisibilidades do ambiente. A ocorrência de aprendizagem já foi observada em parasitoides braconídeos, tais como *Aphidius ervi* Haliday (Du *et al*., 1997), *Biosteres arisanus* (Sonan) (Dukas & Duan, 2000) e *Diachasmimorpha longicaudata* (Ashmead) (Segura *et al.*, 2007).

O comportamento de busca em inimigos naturais pode ser influenciado pela experiência durante diferentes fases do seu ciclo de vida, sendo que, algumas pistas são aprendidas no estágio imaturo (condicionamento pré-imaginal), as quais são subsequentemente manifestadas no adulto (Corbet, 1985; Turlings *et al*., 1993). Neste sentido, Rains *et al*. (2006) observaram que o estímulo olfativo ao composto de 3-octanona, recebido na fase larval pode influenciar o comportamento quimiotáxico de adultos de *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae). Fato semelhante já tinha sido relatado por Sujii *et al.* (2002), os quais observaram que o parasitismo de *T. podisi* provenientes de ovos de *E. heros* foi maior nesta espécie do que em *N. viridula* e *Acrosternun aseadum* Rolston (Hemiptera: Pentatomidae). Segundo a hipótese dos autores, o parasitismo é influenciado pelo hospedeiro de origem, podendo este fornecer pistas químicas que teriam influência direta na escolha e desempenho reprodutivo do parasitoide.

De acordo com Corbet (1985), as informações obtidas na fase imatura podem ser transmitidas ao adulto através da Hipótese do Legado Químico, o qual prediz que compostos químicos presentes no ambiente de desenvolvimento larval estariam presentes na hemolinfa dos insetos ou na camada externa do hospedeiro. Esta hipótese foi posteriormente confirmada por estudos realizados com parasitoides, demonstrando que a escolha do adulto é influenciada por compostos químicos presentes sobre o exoesqueleto do hospedeiro no qual o parasitoide se desenvolveu (Vet & Dicke, 1992; Van Emden *et al*., 1996; Storeck *et al*., 2000).

Tal hipótese apresentada por Corbet (1985) foi também comprovada por Bjorksten & Hoffmann (1998) em *T. brassicae* (Hymenoptera, Trichogrammatidae) e Dukas & Duan (2000), com *B. arisanus*. Em ambos os estudos, os parasitoides de ovos associaram os voláteis de folhas e frutos presentes no ambiente de criação com a presença do hospedeiro.

Matthews & Matthews, (2010) observaram que adultos de *Hyssopus pallidus* (Askew) (Hymenoptera: Eulophidae), um parasitoide de larvas de *Cydia pomonella* L. (Lepidoptera: Tortricidae) que não haviam sido expostos ao extrato de maçã na fase imatura não apresentaram resposta significativa a este odor, mas quando permaneceram em contato com o extrato durante todo o período larval, responderam positivamente ao tratamento. Comportamento semelhante associado à Teoria do Legado Químico foi constatado por Tognon *et al*. (2013) em estudo sobre a avaliação da aprendizagem e memória de *T. podisi*, pois o odor de capim-limão [*Cymbopogon citratus* (DC.) Stapf. (Poales: Poaceae)] o qual é descrito como repelente para vários insetos, não atraiu fêmeas de *T. podisi* de forma inata. No entanto, quando o mesmo permaneceu em contato durante todo o desenvolvimento imaturo do parasitoide, alterou a orientação quimiotáxica de fêmeas desta espécie, sendo que este comportamento se manteve por até 72 horas.

A aprendizagem dos parasitoides pode também ocorrer na fase adulta (Papaj & Lewis, 1993). Fêmeas experientes de *T. basalis*, ou seja, que estiveram em contato com semioquímicos do hospedeiro (*N. viridula*), foram mais atraídas a este odor do que as inexperientes, evidenciando que aquelas são capazes de ajustar o comportamento de busca a sítios de oviposição, de acordo com experiências do passado (Dauphin *et al*., 2009). Estudando estes mesmos organismos, Peri *et al.* (2006) constataram que a memória de *T. basalis* foi mantida por até 23 horas após o primeiro contato deste inseto com pistas químicas deixadas no substrato pelo andar de *N. viridula*.

Outras espécies, como *Eupelmus vuilleti* Crawford (Hymenoptera: Eupelmidae) (fêmeas) mantiveram a memória por seis e sete dias quando expostas aos voláteis de *Bruchidius atrolineatus* (Pic) (Coleoptera: Bruchidae) e aos odores de sementes hospedeiras do inseto herbívoro, respectivamente (Cortesero *et al*., 1995). Em braconídeos, como *Cotesia congregata* (Say), foi verificado que as fêmeas mantiveram a memória por até sete dias ao odor de cerejeira silvestre, a qual estava associada ao seu hospedeiro *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Kester & Barbosa, 1991). Para *M. croceipes*, a aprendizagem a três odores específicos (extratos de baunilha, de laranja e cariofileno), oferecidos juntamente com a alimentação, mas isolados entre si, durou aproximadamente cinco dias (Takasu & Lewis, 1996).

Conforme previamente descrito, *T. basalis* e *T. podisi* são agentes de controle com significativa importância em agroecossistemas porém, embora sendo estas consideradas espécies generalistas, mostram uma preferência por determinadas espécies de percevejos. Por exemplo, *T. basalis* apresenta preferência a ovos do percevejo verde (*N. viridula*) (Sujii *et al*., 2002), enquanto *T. podisi* por ovos do percevejo-marrom, *E. heros* (Sujii *et al*., 2002; Tognon *et al*., 2014).

Para Vet & Dicke (1992) e Vinson (1998) a orientação de parasitoides pode ter uma estreita relação com pistas associadas aos voláteis do hospedeiro de origem, adquiridos através da aprendizagem durante o desenvolvimento do estágio imaturo, permanecendo assim com uma memória olfativa após a emergência, fato que comprova a preferência de certos parasitoides por determinados hospedeiros.

A inexistência de parasitismo em ovos de *Tibraca limbativentris* Stål (Hemiptera: Pentatomidae) por fêmeas de *T. podisi* oriundas de *E. heros*, foi observado por Tognon *et al*. (2014) sendo este fator, conforme descrito anteriormente por Matthews & Matthews (2010), um condicionamento pré-imaginal. Este condicionamento pode influenciar a preferência por determinadas pistas (Turlings *et al*., 1993) o que, segundo a hipótese descrita por Cobert (1985) pode ocorrer, quando um parasitoide é criado por várias gerações em um único hospedeiro. *Trichogramma pretiosum* Riley (Hymenoptera: Trichogrammatidae) prefere ovos de *Anagasta kuehniella* Zeller (Lepidoptera: Pyralidae), hospedeiro da qual provieram, porém após passarem uma geração em *S. frugiperda*, preferiram parasitar ovos deste segundo lepidóptero (Goulart *et al*., 2011).

2.5 Utilização de semioquímicos na manipulação de parasitoides em ambientes agrícolas

Visando a integração dos conhecimentos relacionado à interação de inimigos naturais com seus hospedeiros, estudos vêm sendo desenvolvidos no sentido de manipular tanto o habitat (Simpson *et al*., 2011ab; Vieira *et al*., 2014) como a neurofisiologia dos insetos benéficos (Lewis *et al*., 1975ab; Hare *et al*., 1997) incrementando o encontro entre estes organismos. Vieira *et al*. (2014), por exemplo, avaliaram o composto sintético (*E*)-2-hexanal (encontrado em secreções de glândulas metatorácicas de *E. heros*) na atratividade e recrutamento de Telenominae na cultura da soja e concluíram que na presença deste composto ocorreu um aumento na abundância de *Trissolcus* spp., mas não no parasitismo na área de estudo. No entanto, Peres (2004) utilizando dispersores (septos de borracha) impregnados com (*E*)-2-hexanal, um composto presente nas glândulas metatorácicas de *E. heros* (Borges & Aldrich, 1992, 1994; Moraes *et al*., 2008), observaram um aumento no número de parasitoides e no parasitismo de ovos de percevejos.

Como tentativa de sincronizar populações no ambiente, uma informação de grande relevância está ligada à dinâmica populacional dos insetos. Em culturas anuais, as quais possuem uma entomofauna usualmente instável, as populações de pragas se estabelecem antes que as dos inimigos naturais, fato que impede aos insetos benéficos um crescimento populacional capaz de diminuir a do hospedeiro abaixo do nível de dano (Ehler & Miller, 1978; Borges *et al*., 1998a; 1998b). Este fato também foi comprovado para populações do percevejo *E. heros* e seus principais parasitoides de ovos, *T. podisi* e *T. basalis*, em culturas de soja na região central do Brasil (Moraes *et al*., 2013). Estas informações demonstram o potencial do uso de semioquímicos em ambientes agrícolas com o intuito de atrair parasitoides antes da população da praga se estabelecer no campo e intensificar o dano na cultura (Vieira *et al*., 2014). Sendo assim, o sucesso do uso de inimigos naturais no meio agrícola depende de dois importantes fatores: atrair os insetos benéficos em sincronia com a população da praga e retê-los no campo (Vinson, 1984; 1985). Portanto, esta técnica pode ser melhorada com a introdução/conservação de espécies vegetais que possuem em seu ciclo reprodutivo uma alta produção de inflorescências, as quais podem fornecer aos inimigos naturais um microclima adequado, presas ou hospedeiros alternativos e alimento tal como néctar e pólen (Ellis *et al*., 2005; Kennedy & Storer, 2003; Landis *et al*., 2000).

Esta nova tática de controle biológico conservativo, chamada atração e recompensa, combina a associação de voláteis atrativos aos parasitoides, cairomônios ou sinomônios, com plantas que ofereçam alimento e refúgio aos inimigos naturais (Khan *et al*., 2008; Simpson *et al*., 2011a). Esta visa um conjunto de práticas para a manipulação ambiental e comportamental dos insetos benéficos criando um ambiente agrícola adequado para recrutar e conservar as populações atraídas em áreas onde se encontra a praga (Simpson *et al*., 2011ab; Khan *et al*., 2008).

Este método tem como sustentação teórica a hipótese em que os predadores e parasitoides controlariam herbívoros de forma mais eficaz em ambientes heterogêneos ao invés de homogêneos, pois teriam maior acesso a recursos, pois a maioria dos himenópteros adultos necessitam de carboidratos como fontes alimentares sendo o néctar ou outras fontes de açúcar eficazes em satisfazer as necessidades biológicas dos mesmos (Bianchi & Wackers, 2008) aumentando tanto sua longevidade quanto a fecundidade (Witting-Bissinger *et al*., 2008).

Estudos em áreas agrícolas já têm mostrado que a presença de plantas com flores pode diminuir problemas ocasionados pelas pragas através do aumento das populações de inimigos naturais (Bianchi & Wäckers, 2008; Ellis *et al*., 2005; Simpson *et al*., 2013), tais como *Lobularia maritima* L. (Brassicales: Brassicaceae) e *Coriandrum sativum* L. (Apiales: Apiaceae).

Pease & Zalom (2010) concluíram que o parasitismo em ovos de *E. conspersus* e *Thyanta pallidovirens* Stal (Heteroptera: Pentatomidae) foi significativamente maior em áreas de cultivo de tomate que continham plantas de *L. maritima* na bordadura da plantação em relação às áreas controle, sem a presença da planta, sendo que as principais espécies coletadas foram *Gyron obesum* Masner, *Trissolcus hullensis* (Harrington), *Trissolcus utahensis* (Ashmead) (Hymenoptera: Scelionidae) e *Oencyrtus johnsoni* (Howard) (Hymenoptera: Encyrtidae).

Aliando manipulação comportamental e do habitat, Simpson *et al*. (2011a) colocaram em prática a técnica “atração e recompensa” realizando um estudo que combinou a aplicação de voláteis que induziram as plantas de milho a produzir compostos atrativos aos parasitoides com plantas de *Fagopyrum esculentum* (Caryophyllales: Polygonaceae), como recompensa. Os autores observaram a presença de parasitoides pertencentes à Eulophidae, Encyrtidae e Scelionidae e a diminuição significativa de larvas de *Helicoverpa* spp. (Lepidoptera: Noctuidae) sobre as plantas de milho.

Essa manipulação comportamental pode também ser a nível fisiológico dos parasitoides em condições de criações massais em laboratórios com o intuito de liberação em programas de controle biológico inundativo (Hare *et al.*, 1997). Conforme Strand & Vinson (1983) já haviam sugerido, a utilização de cairomônios que atuam no reconhecimento e aceitação de hospedeiros em criações massais de inimigos naturais pode promover o aceite de novos hospedeiros, direcionando uma vespa a parasitar um determinado hospedeiro alvo. Um claro exemplo foi apresentado por Hare *et al*. (1997) em estudos com *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), onde vespas criadas em *Aspidiotus nerri* Bouché (Hemiptera: Diaspididae) aumentaram o parasitismo em um novo hospedeiro, *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae) após serem mantidas em contato com um composto cairomonal sintético deste inseto.

2.6 Referências bibliográficas

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3 ARTIGO 1

Is it possible to manipulate egg parasitoids' preference for a target host?*

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Title: Is it possible to manipulate egg parasitoids' preference for a target host?

Roberta Tognon, Josué Sant'Ana, Luíza Rodrigues Redaelli, Augusto Leal Meyer

Department of Crop Protection, PPG-Fitotecnia, Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 7712, 91540-000 Porto Alegre, RS, Brazil.

Corresponding author:

Email address: roberta.tognon@ufrgs.br (Roberta Tognon)

Abstract

Parasitoids host-selection is mainly mediated by chemical cues, which can be adjusted by experience, changing its innate behavior. The learning process can enhance the insect's probability to find suitable hosts, increasing their ability for a new environment condition. Therefore, the objective of this study was to determine if immature experience on eggs and volatiles from host eggs, has influence on parasitism and chemotaxic behavior of *Telenomus podisi* Ashmead and/or *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae). Firstly, both wasp species were submitted to a parasitism choice test with eggs of *Euschistus heros* (Fabricius), *Piezodorus guildinii* (Westwood) and *Nezara viridula* L. (Hemiptera: Pentatomidae). The subsequent parasitoid generation from each host was also bioassayed on the same way. Moreover, the innate behavior and experience of *T. podisi* was recorded in olfactometer "Y" to volatiles from pentamomids' egg extracts, in order to study its learning and memory abilities. *Telenomus podisi* parasitized more eggs from its original host, however, in *T. basalis* the last host rearing had no influence on its choice, showing a specific preference for *N. viridula* egg masses. Naïve *T. podisi* females responded positively to *E. heros* and *P. guildinii* and negatively to *N. viridula* egg volatiles. However, they learned to respond to its last cue after experience and memorized it for, at least, 24 hours. We believe that is possible to manipulate *T. podisi* behavior through experience with host egg's kairomones.

Key words *Telenomus podisi*, *Trissolcus basalis*, Pentatomidae, Scelionidae, learning, parasitoid manipulation

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Introduction

The egg parasitoid host selection is mediated by two main steps, host location and host recognition using long and short-range cues, respectively (Godfray 1994; Vinson 1998). The patterns of parasitoid foraging are determined by environment factors, genetic physiology and experiences (Turlings et al. 1993; Vet et al. 1995), which remain two important questions on what is the value of learning and what is the function of behavioral variability for natural enemies as parasitoid wasps (Papaj and Vet 1990; Vet and Dicke 1992).

In the 'variable-response model' proposed by Vet et al. (1990), wasps have innate responses to each series of chemical cues used for locating their hosts, so the insect variability to respond for each stimulus should be related to their potential benefits. Egg volatiles, e.g., are a reliable and relevant signal mediating egg parasitoid search and recognition behavior in short distances (Bin et al. 1993; Borges et al. 1999; Michereff et al. 2016; Tognon et al. 2014; 2016).

However, the dilemma acquired new evidence through the reliability-detectability theory. Female parasitoids are under selection pressure to efficiently intensify their limited time/resources on the host location (Vet and Dicke 1992). Cues from hosts may be highly reliable, but are less detectable at long-range, compared to volatiles from plants, which have a much larger amount. Besides, the environmental complexity is high under natural conditions and the wasps make use of their learning capacity, which will let them overcome the environmental barrier (Wäschke et al. 2013).

Learning play a key role in parasitoid behavior (Vet and Dicke 1992; Takasu and Lewis 2003) and a considerable degree of brain plasticity, explained as the competence of one organism to change its physiology in response to environment conditions, manifested by an individual insect, may have a significant impact on evolutionary changes in host use (van den Berg et al. 2011; Wäschke et al. 2013). Adjusting their behavior to certain chemical compounds would help parasitoids to take advantage for finding easer suitable hosts (Turlings et al. 1993).

Telenomus podisi Ashmead and *Trissolcus basalis* Wollaston (Hymenoptera: Scelionidae) are known as the most important egg parasitoids of pentatomids including, *Euschistus heros* (Fabricius), *Piezodorus guildinii* (Westwood) and *Nezara viridula* (L.), which consist a pest complex on soybean crops in Brazil (Corrêa-Ferreira and Moscardi 1995). Both wasp species are attracted to a wide range of pentatomids (Austin et al. 2005; Tillman 2011), however specific studies have observed a *T. podisi* preference for *E. heros* (Pacheco and Corrêa-Ferreira 1998; Tillman 2010; Tognon et al. 2014), while *T. basalis* prefers *N. viridula* egg masses (Sujii et al. 2002; Tillman 2011).

Previous researches have observed changing in preferences related to origin host, i.e., when the host of *T. podisi* was *E. heros*, the parasitism was greater on this stink bug rather than *Tibraca limbativentris* Stål (Hemiptera: Pentatomidae), similar behavior was registered when wasps were reared on *T. limbativentris*, parasitizing more the last species (Tognon et al. 2014). Nevertheless, recently Tognon et al. (2016) reported a different effect of synthetic volatiles from *Euschistus conspersus* Uhler and *Halyomorpha halys* Stål

(Hemiptera: Pentatomidae) egg masses on scelionids search behavior and parasitism, being the first attractant and the second, repellent to *T. podisi*. In a subsequent study, experienced wasps with volatiles from *H. halys*, in the immature stage, resulted in a successful parasitism on this host (R. Tognon et al. unpublished yet).

Hare et al. (1997) demonstrated that *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), a parasitoid of the California red scale *Aonidiella aurantii* (Maskell) (Hemiptera: Diaspididae), exposed to a host kairomone prior being released in the field, improves its parasitoid activity. Thus, the behavior manipulation of parasitoids is a new technique for allowing the use of natural enemies in biological control programs (Meiners and Peri 2013). Therefore, the objective of this study was to observe if experience in egg masses from specific host and their volatiles have influence on parasitism and chemotaxic behavior of *T. podisi* and/or *T. basalis*.

Materials and methods

We reared insects and carried out bioassays under controlled condition chamber (26 ± 1°C, 65 ± 10% RH, 14h photophase), in the Ethology and Insect Chemical Ecology Laboratory, at the Federal University of Rio Grande do Sul (UFRGS).

Acquisition and insects rearing

We stablished an *E. heros* colony from egg masses supplied by Genetic Resources and Biotechnology Laboratory (EMBRAPA/Cenargen), Brasilia, DF, Brazil.

The adults were reared in 19 x 25 x 19 cm plastic cages, supplied with water in a glass shell vial with a cotton wick and fed with fresh green beans, soybean, sunflower and peanut seeds as recommended by Borges et al. (2006). Paper towels served as an oviposition substrate. Eggs were collected daily and kept under the same conditions in separate nymphal rearing cages or removed for using in bioassays. Newly adults were removed daily from the immature cages and transferred to separate ones containing recently emerged insects. Each cage contained no more than 50 adults and food was replaced twice a week. *Piezodorus guildinii* and *N. viridula* adults were collected on soybean field at Experimental Agriculture Station of UFRGS (EEA-UFRGS) in Eldorado do Sul, RS, Brazil. These two last species were kept under same laboratory conditions as described for *E. heros*. *Telenomus podisi* and *T. basalis* (confirmed by Dr. Valmir Antônio Costa from Biological Institute of São Paulo, Brazil) were obtained from EMBRAPA/Cenargen. We reared them in laboratory on the same host as provided, *E. heros*. Moreover, a second group of *T. podisi* (also confirmed by Dr. Costa) was collected on soybean field at EEA-UFRGS in *P. guildinii* eggs. Wasps were kept in glass tubes (7.5 x 1.3 cm) sealed with Parafilm® (Bemis Flexible Packaging, Neenah, WI) and fed with a drop of honey. In our experiments, only females (approximately 48 h old), previously paired with males, were used. Each wasp was tested only once.

Stink bug egg extracts

Mated females of *E. heros*, *P. guildinii* and *N. viridula*, were separated from males and kept in different cages with food, water, and paper toweling for oviposition. Eggs (12-24 h old) were removed from the paper using forceps, placed in glass vials (4 mL clear vial, W/PTFE cap; Sigma-Aldrich St. Louis, Missouri, USA) and weighed. Enough hexane (99%, Sigma-Aldrich) (~ 1 mL) was added to ~1 g quantities of eggs to cover them. After 5 min, the solvent was transferred by a glass syringe to another clean glass vial. The samples were kept at -4 ºC.

Bioassays

Host preference of *T. podisi* and *T. basalis*

Egg masses of *E. heros*, *P. guildinii* and *N. viridula* (n = 10 eggs/each stink bug species) were glued onto a 1 x 1.5 cm filter paper strip using double-sided sticky tape, and placed randomized in each assay, in a 7.5 x 1.3 cm glass tube containing a drop of honey. After that, one 48 h old female of either *T. podisi* (from *E. heros* or *P. guildinii*) or *T. basalis* (from *E. heros*) was placed, for 3 hours, into a tube sealed with Parafilm® (Bemis Flexible Packaging, Neenah, WI). Female wasps emerged from each host (first generation) were used for an additional multiple choice parasitism test as previously described. We dissected the eggs which nether parasitoids nor nymphs emerged. Stink bug egg masses not exposed to any parasitoids were set into 7.5 x 1.3 cm glass tube as control treatment. We carried out at least 20 replicates for each bioassay.

Olfactometry

Euschistus heros eggs (24 h old) were offered to *T. podisi*. After 24 h, the masses were placed in a glass Petri dish, rinsed with 99% hexane for 5 min, air-dried and coated with 5 µL of egg extracts from *P. guildinii* or *N. viridula.* This procedure was done each 3 days, for approximately 12 days, until 2 days before emergence. Chemotactic response of these experienced parasitoids females (24 h old) to these treatments was evaluated in a two-choice test using a horizontally positioned Y-tube glass olfactometer (1.4-cm diameter), with a 16-cm basal arm and bifurcated at a 60° angle into two 19-cm arms. Air flow was 0.8 L/min through an air pump connected to a flow meter and a humidifier. Each female was placed individually in a glass tube (5 mL), fed with a drop of honey for 24 hours before the bioassay.

A single experienced wasp (either with *P. guildinii* or *N. viridula* eggs extract) was introduced into the Y-tube and allowed to choose between a piece of filter paper (1 x 2 cm, P5 Fisherbrand®, Fisher Scientific, Marshalltown, IA, USA) containing 5 µL of *P. guildinii* egg extracts (13 eggs equivalent, EE) or *N. viridula* (4.6 EE) in contrast with 5µL of hexane (control). Responses of females from *E. heros* eggs without contact with extracts (inexperienced insects), were submitted to the same treatments, as a control, as well as, to *E. heros* extract (10 EE). Furthermore, tested females were kept separately in a glass tube (5 mL), fed with a drop of honey, to be bioassayed in the olfactometer again 24 hours after the first exposition (memory test).

All bioassays were conducted during the photophase period and under fluorescent bulb (9 W, luminance = 290 lux). After three insects tested, the odor sources were switched to the opposite side, to avoid any positional bias. Glassware was rinsed for each of the six replicates with fragrance-free liquid soap followed by distilled water and dried in a convection oven at 100 ^{o}C. Each insect was given 10 min to make a choice of arms in the olfactometer. Parasitoids that moved at least 3 cm into one branch arm and remained there for at least 60 s, were recorded as responsive. If no choice was made in 10 min, the assay was concluded and the insect considered non-responsive, being excluded from statistical analysis. We carried out, at least, 40 replicates for each test.

Parasitism learning with egg extracts

Telenomus podisi females (24 h old) experienced in *P. guildinii* or *N. viridula* egg extract were submitted to parasitism as a multiple-choice test. Experienced wasps were kept into a glass tube (7.5 x 1.3 cm), with a drop of honey, sealed with parafilm and offered *E. heros*, *P. guildinii* and *N. viridula* egg masses (10 eggs each masse) as previously described in the first parasitism bioassays. Inexperience females (from eggs of *E. heros* not rinsed) were the control treatment. We carried out at least 20 replicates each choice experiment.

Statistical analyses

The first choice on the olfactometer was analyzed by χ^2-tests ($P < 0.05$). Means of parasitized eggs were compared within treatments by Kruskal-Wallis H-test ($P < 0.05$) with BioEstat® 5.0 (Ayres et al. 2007).

Results

Host preference of *T. podisi* and *T. basalis*

Telenomus podisi from *E. heros* (lab colony) parasitized more the original host eggs (73.66%) than *P. guildinii* (41.33%) (H = 10.92; df = 1; $P = 0.001$). The emerged females from *P. guildinii* changed their preference after one generation on this last host, parasitizing more *P. guildinii* than *E. heros* ($P < 0.05$). In both tests, we did not observed parasitism on *N. viridula* egg masses (Figure 1).

However, the parasitism of *T. podisi* from *P. guildinii* (collected on field) was greater on its original host (81.15%) than on *E. heros* (45.76%) and *N. viridula* (0.38%) (H = 33.32; df = 2; $P < 0.0001$). Emerged wasps from *E. heros* preferred parasitize *E. heros* eggs (71.38%) rather than *P. guildinii* (25.5%) ($P < 0.05$). For *N. viridula* we observed only 0.83% of parasitism (Figure 1). We did not carry out parasitism tests using *T. podisi* emerged from *N. viridula* due to the low parasitism rates and only male's emergence. *Trissolcus basalis* from *E. heros* (lab colony), *P. guildinii* or *N. viridula* (first generation) had preference to *N. viridula* eggs ($P < 0.05$) (Figure 2).

Olfactometry

Naïve *T. podisi* were significantly more responsive to *E. heros* egg extract (60.46%) rather than control (30.23%) ($\chi^2 = 8.667$; df = 1; $P = 0.0066$). Wasps (experienced and not) responded positively to *P. guildinii* egg extract when contrasted with control, either with 24 or 48 h old ($P < 0.05$) (Figure 3).

Inexperienced wasps did not show a significant choice between *N. viridula* egg volatiles versus the hexane either with 24 h or 48 h old ($P > 0.05$). However, 24 hours old females changed their behavior after experience, being 69.04% more attracted to extract than to hexane (21.42%) ($\chi^2 = 21.053$; df = 1; P = 0.0001). Although after 48 h, they have showed a similar behavior than those naïve ones (Figure 4).

Parasitism learning with egg extract

Telenomus podisi females without experience parasitized more *E. heros* (79.6%) than *P. guildinii* eggs (19.5%) (H = 21.2418; df = 1; *P* < 0.0001), but when they were experienced on *P. guildinii* egg extract, they parasitized more this host (53.2%) than *E. heros* (30.5%) (H = 4.1891; df = 1; *P* = 0.0407). In both tests, we did not observed parasitism on *N. viridula.*

Even when the wasps were experienced on *N. viridula* extract, they did not parasitize it successfully, it was observed only in *P. guildinii* (20%) and *E. heros* (35%) (H = 1.8326; gl = 1; *P* = 0.1758). Interestingly, we found a high *N. viridula* egg mortality of (60.5%) exposed to experienced females, the same was not observed in the other treatments (Table 1).

Discussion

Our results showed that parasitism of *T. podisi* increases in *E. heros* or *P. guildinii*, depending on the host where it came from. This finding is reminiscent to the pre-imaginal condition discussed in chemical legacy hypothesis by Corbet (1985), which suggests that larval chemical environment found in the hemolymph or on the external layer of host egg have influence on parasitoid choice. It was already known that *T. podisi* is more likely to parasitize the host in which it development occurred, preserving a chemical legacy from the odor that its larvae were exposed (Tognon et al. 2014). In Selionidae species and some other parasitoids, chemical environmental from original host is an essential reminder which lead to faster host finding and/or more intensive searching (Meiners and Peri 2013; Wäschke et al. 2013), thus learning allows parasitoids to focus on most reliable cues (Papaj and Lewis 1993; Vet et al. 1990).

On the other hand, *T. basalis* showed a specific preference to *N. viridula* egg masses, independently of the host from which the wasp emerged, i.e., an innate behavior that is not modified by experience during immature stage. A similar result was reported by Goulart et al. (2011) to *Telenomus remus* Nixon (Hymenoptera: Scelionidae) in which even after several generations on *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), exhibit a preference for *Spodoptera cosmioides* (Walker) (Lepidoptera: Noctuidae) egg masses.

These observations drive us to a relevant topic, i.e., the relationship between host and parasitoid involves a series of interactions outside and inside the host egg, primarily mediated by chemical factors, that might or not be related to the origin host.

A successful parasitoid-host interaction occurs only if parasitoids are able to release enough toxins to prevent host embryonic growing, without being affected by its immune system, and get necessary nutrients for their development (Cônsoli et al. 2001; Strand et al. 1986). Therefore, host population can be classified as either susceptible, providing all of requirements of a given parasitoid species, or resistant, capable of avoiding attack or eliminating parasitoid embryo (Strand and Pech 1995).

Strand et al. (1985, 1986) evidenced that *Heliothis virescens* (F.) (Lepidoptera: Noctuidae) is susceptible to *Telenomus heliothidis* Ashmead (Hymenoptera: Scelionidae), that injects in the host an arrestment chemical present around its eggs, which acted together with a substance produced by specific cells, called teratocytes, from extraembryonic parasitoid's tissues, resulting in host necrosis. Teratocytes produce enzymes that could help the young larva to digest the contents of the host, facilitating absorption of host nutrients by parasitoid larva (Cônsoli et al. 2001; Strand et al. 1985, 1986). Interestingly, *T. basalis* has specific teratocyte cells that act in *N. viridula* necessary to enable the parasitoid success (Cônsoli et al. 2001).

However, interactions between hosts and parasitoids are also associated to an ancestry history between organisms. Many laboratory and field researches showed a clear relation between *T. podisi*/*E. heros* and *T. basalis*/*N. viridula* (Borges et al. 1999; Sujii et al. 2002; Tillman 2011). Different parasitoids species behavior could be explained and explored by phylogenetic and ancestry host (Taekul et al. 2014). Geographical and phylogenetic data claim that *E. heros* and *P. guildinii* are native species at Neotropical Region (Panizzi and Slansky 1985a; 1985b; Panizzi et al. 2000) and *Telenomus podisi* has the same prevalent area, including the South Neartic Region (HOL). Furthermore, the Ethiopian Region in eastern Africa is the most likely point of *N. viridula* origin (Jones 1988). A revision made by Clarke (1990) revealed that *T. basalis* was first imported into Australia from Egypt in 1933 and after that, the wasp was sporadically imported and release from Australia to several countries including Latin and North America to control *N. viridula*, considered an invasive stink bug. Thus, we may conclude that wasps' preference can be also related to evolutionary and ecological-adaptive behavior.

In our study, parasitism on *N. viridula* by *T. podisi*, without experience, is critically low or absent and hatched nymphs is high, comparing to control (unexposed eggs). In fact, wasps do not appear to recognize *N. viridula* as a host, probably due to chemicals and/or physicals external and internal barrier. It was already observed an external barrier by *H. halys* eggs to the scelionids *T. podisi* and *Trissolcus erugatus* Johnson which were not able to parasitize it (Tognon et al. 2016).

Conversely, eggs' mortality observed when exposed to females experienced with *N. viridula* extract was extremely high (60.5 %), comparing to control (7.61 %). Thus, we may infer that there was a chemical interaction between these two organisms. For instance, after a wasp recognizes an external egg, it must do an internal chemical recognition through sensilla present in the ovipositor (Vinson 1991). Therefore, we hypothesize first, that females probed the internal host (without oviposition) and released substances which caused necrosis in host embryo; second, that female released its egg inside the host, but did not have enough or specific teratocytes for its full larvae development on *N. viridula* and third, egg host did not have appropriate nutrients to parasitoids developmental requirements or contained deterrents substances that enable parasitoids' growing.

We observed an innate response of *T. podisi* to *E. heros* and *P. guildinii* extracts and none to *N. viridula*. The egg extracts from *P. guildinii* and *N. viridula* exposed to *T. podisi* during its development, influenced either chemotactic behavior and/or parasitism. The volatiles probably got contact with parasitoids' larvae through aeropyles of egg, very small channels, responsible for the gas exchanges which lead a behavioral change on the wasps (Cobben 1968; Trougakos & Margaritis 2002).

Telenomus podisi is a very learning flexible species. It was firstly reported responding to extract of lemongrass oil [*Cymbopogon citratus* (DC.) Stapf. (Poales, Poaceae)] (Tognon et al. 2013) and later, to *H. halys* egg aldehydes (R. Tognon unpublished yet), after immature experience, both considered repellent compounds.

As a matter of fact, experience by *N. viridula* extract led learning in *T. podisi* making it overcome the external chemical barrier imposed by the host and recognizing it as a potential one. This observation is supported by the high amount of egg mortality exposed to experienced females, as previously discussed, because such fact was not observed in control treatment.

Surprisingly, the experience with *N. viridula* conducted wasps to memorize the odor until, at least, 24 h. In *T. basalis* it was already reported a 72 h memory when females were exposed to chemicals residues left by *N. viridula* on substrate (Colazza et al. 1999; Peri et al. 2006). In an ecological context, memory would enable natural enemies to have enough time to find a suitable host easer, increasing their biological fitness and parasitism success (Peri et al. 2006; Turlings et al. 1993).

Parasitoids are complex organisms that display a great deal of adaptive plasticity to circumstances, in many cases, those conditions, are state-dependent (Godfray 1994). In this study, *T. podisi* learned cues from a new host and the experience increased searching potentials, parasitism or parasitism attempt. The results are according with Turlings et al. (1993), which claimed that experience can influence responses in two different ways, inducing a general increase in female responsiveness (i.e., priming) and altering female's preference for specific host's cues.

Learning abilities registered here can be related to the brain lobes formation during larval stage. Holometabolous insects have a series of transformations resulting in changes in the nervous system (Barron & Corbet 1999). There are not brain development studies with scelionids, however in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), parts of the mushroom body responsible for the storage of chemosensory information, remain intact during metamorphosis, potentially allowing learning to be maintained from larvae to adults (Armstrong et al. 1998).

Finally, the results here reported have a potential use in the biological pest control, since the parasitoid manipulation is an open and new opportunity for improving this tool. We believe that is possible to manipulate *T. podisi* behavior through experience. The use of kairomones on recognition can promote new hosts acceptance (Hare et al. 1997; Strand and Vinson 1983). Thus, these results offer additional support for studies of using learning to manage natural enemies in field conditions.

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Figure captions

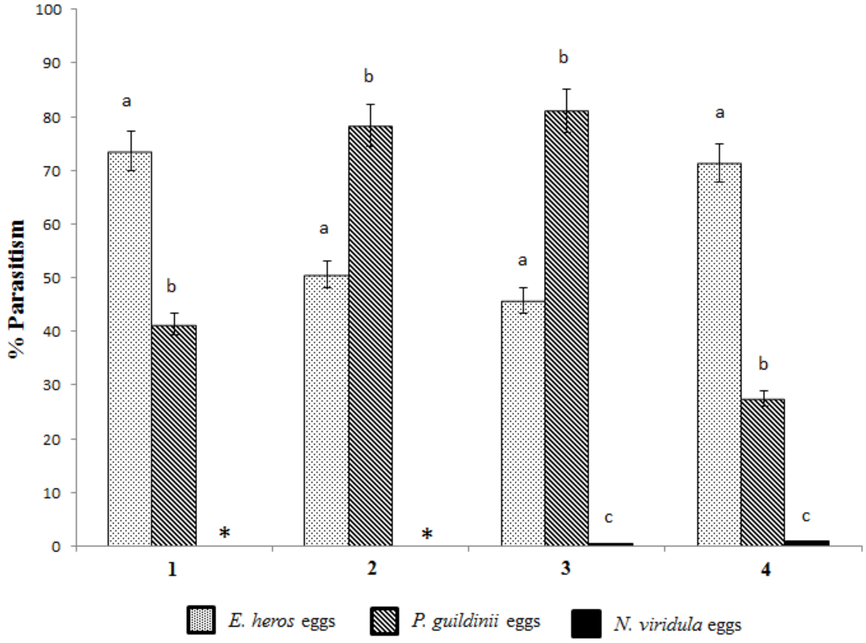
Fig 1 Parasitism percentage (±SE) of *Telenomus podisi* from *Euschistus heros* (1) and those emerged on *Piezodorus guildinii* as 1st generation (2); *T. podisi* from *P. guildinii* (3) and those emerged on *E. heros* as 1st generation (4) submitted to a multiple-choice test with *E. heros*, *P. guildinii* and *Nezara viridula* eggs. Bars followed by different letters, within parameters in each number, are significantly different (Kruskal-Wallis, $P < 0.05$). *No egg parasitized

Fig 2 Parasitism percentage (±SE) of *Trissolcus basalis* from *Euschistus heros* (1) and those emerged on *Piezodorus guildinii* (2) and *Nezara viridula* (3) as 1st generation submitted to a multiple-choice test with *E. heros*, *P. guildinii* and *Nezara viridula* eggs. Bars followed by different letters, within parameters in each number, are significantly different (Kruskal-Wallis, $P < 0.05$).

Fig 3 First choice of *Telenomus podisi* females 24 h and 48 h old without (innate behavior) and with experience to volatiles from *P. guildinii* egg extract, tested in Y-tube olfactometer to volatiles of eggs versus hexane (control). Numbers represent the total of responsive insects to the treatments. Numbers in parentheses represent the nonresponsive (NR) wasps . Value followed by an asterisk differ significantly (χ^2, $P < 0.05$) of hexane within each treatment (inexperienced or experienced females).

Fig 4 First choice of *Telenomus podisi* females 24 h and 48 h old without (innate behavior) and with experience to volatiles from *N. viridula* egg extract, tested in Y-tube olfactometer to volatiles of eggs versus hexane (control). Numbers represent the total of responsive insects to the treatments. Numbers in parentheses represent the nonresponsive (NR) wasps. Value followed by an asterisk differ significantly (χ^2, $P < 0.05$) of hexane within each treatment (inexperienced or experienced females).

Fig 1


% Parasitism
100
90
80
70
60
50
40
30
20
10
0
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b
*
a
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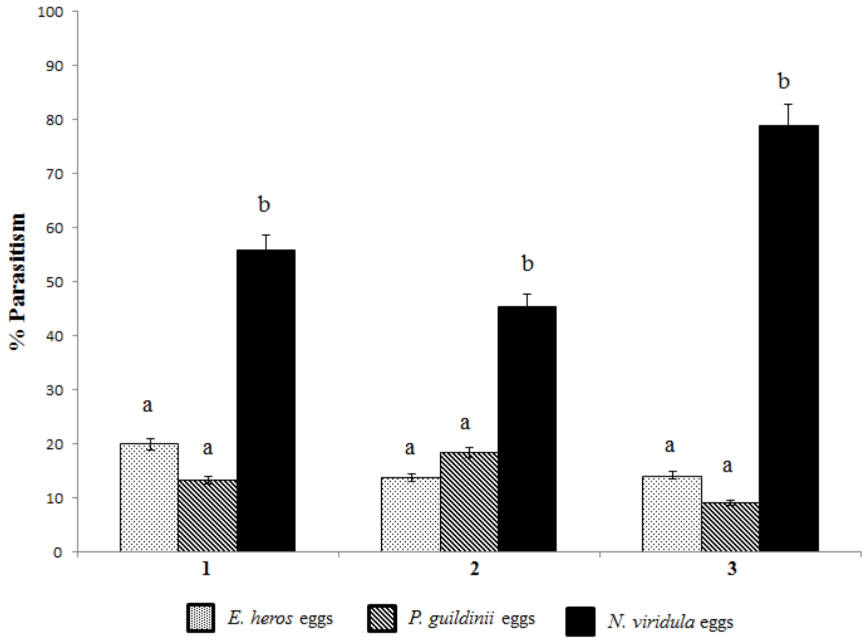
*
a
b
c
a
b
c
1
2
3
4
E. heros eggs
P. guildinii eggs
N. viridula eggs

Fig 2


% Parasitism
100
90
80
70
60
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40
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20
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0
a
a
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b
a
a
b
1
2
3
E. heros eggs
P. guildinii eggs
N. viridula eggs

Fig 3

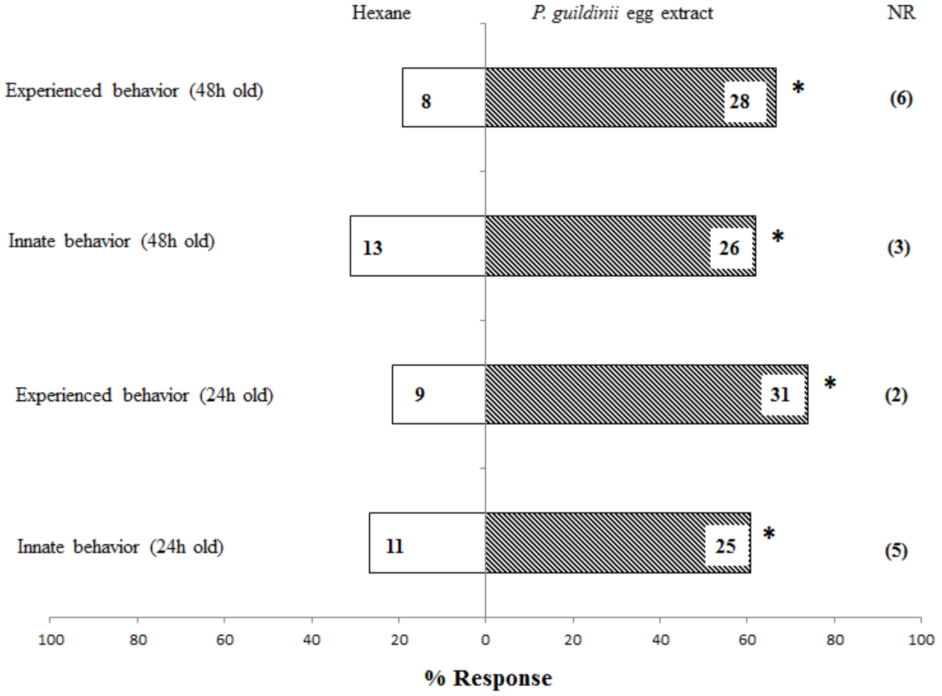
Hexane
P. guildinii egg extract
NR
Experienced behavior (48h old)
8
28
*
(6)
Innate behavior (48h old)
13
26
*
(3)
Experienced behavior (24h old)
9
31
*
(2)
Innate behavior (24h old)
11
25
*
(5)
100
80
60
40
20
0
20
40
60
80
100
% Response

Fig 4

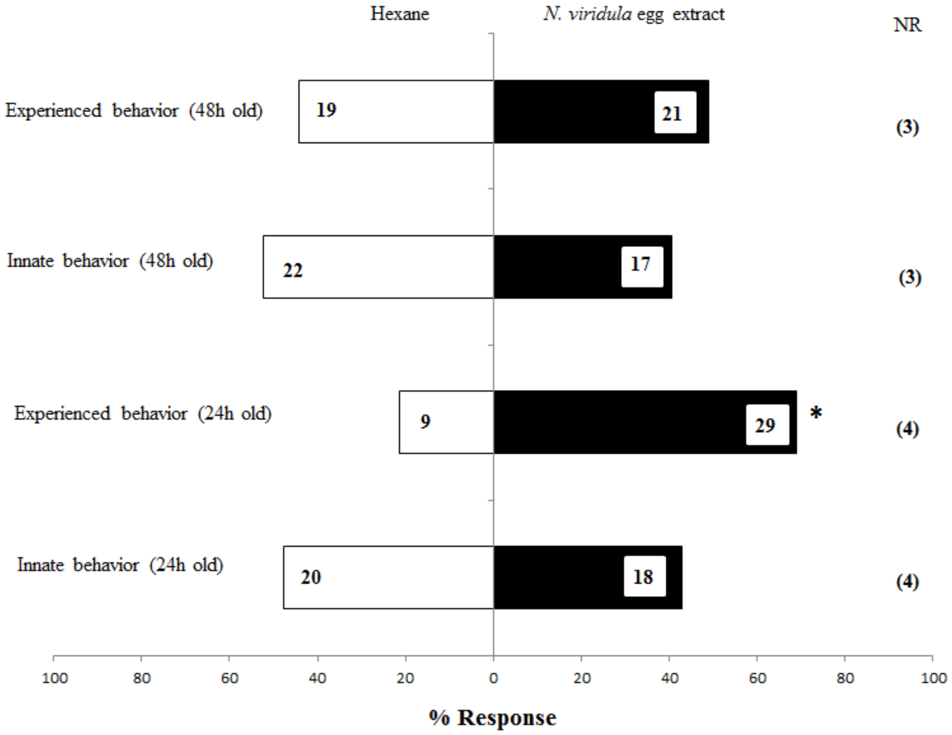
Hexane
N. viridula egg extract
NR
Experienced behavior (48h old)
19
21
(3)
Innate behavior (48h old)
22
17
(3)
Experienced behavior (24h old)
9
29
*
(4)
Innate behavior (24h old)
20
18
(4)
100
80
60
40
20
0
20
40
60
80
100
% Response

Table 1 Parasitism of *Euschistus heros* (EH), *Piezodorus guildinii* (PG) and *Nezara viridula* (NV) eggs by *Telenomus podisi* naïve females (1) experienced on *P. guildinii* (2) and *N. viridula* (3) egg extracts; and unexposed eggs (4)

Treatments		No. exposed eggs	% of parasitized eggs*	% of parasitoid emergence*	% of nymphal emergence*	% of egg mortality* [a]
1	EH	210	79.6a	78.5a	16.2a	4.2a
	PG	210	19.5b	18.1b	79.1b	1.4a
	NV	210	0	0	92.3b	7.7a
2	EH	220	30.5a	29.6a	65.4ab	4.1a
	PG	220	53.2b	53.2b	46.4b	0.4a
	NV	220	0	0	90.4a	9.6a
3	EH	200	35a	34a	63ab	2a
	PG	200	20b	19.3b	78b	2a
	NV	200	0	0	39.5a	60.5b
4	EH	230	-	-	97.3a	2.6a
	PG	220	-	-	96.91a	3.18a
	NV	220	-	-	97.27a	2.8a

*Numbers followed by different letters are significantly different within each treatment/condition/column at Kruskal-Wallis $P < 0.05$

[a] These include the percentage of eggs from which neither parasitoids nor nymphs emerged

4 ARTIGO 2

Volatiles from *Euschistus heros* egg masses and their kairomonal effect on *Telenomus podisi* *

*****Artigo formatado conforme as normas da revista Entomologia Experimentalis et Applicata

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Title: Volatiles from *Euschistus heros* egg masses and their kairomonal effect on *Telenomus podisi*

R Tognon[1], J Sant'Ana[1], R.A. Laumann[2], M. Borges[2], MC Blassioli-Moraes[2], LR Redaelli[1]

[1]Department of Crop Protection, PPG-Fitotecnia, Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 7712, 91540-000 Porto Alegre, RS, Brazil.

[2] Genetic Resources and Biotechnology Laboratory (EMBRAPA/Cenargen), 70849-970, Brasília, DF, Brazil

Corresponding author:
Email address: roberta.tognon@ufrgs.br (Roberta Tognon)

Short title: Volatiles from *Euschistus heros* egg masses

Key words: egg kairomones, chemical identification, Scelionidae, *Telenomus podisi*, Pentatomidae, biological control improvement, behavioral manipulation, volatiles compounds

Abstract

Telenomus podisi Ashmead (Hymenoptera: Scelionidae) is the most important egg parasitoid of *Euschistus heros* (Fabricius) (Hemiptera: Pentatomidae) and its successful parasitism is related to their searching ability to find suitable hosts under a complexity chemical environment using host reliable cues. Thus, the objective of this study was to elucidate chemical substances on external layer of *E. heros* eggs and report its potential kairomonal activity on *T. podisi*. We tested wasp females in olfactometer system to egg extracts and synthetic compounds resulted from chemical identification. The synthetic blend was also evaluated in parasitism tests under laboratory and semi-field conditions. We identified 32 substances from egg extracts, including terpenes, aldehydes, alkanes and esters. Among those compounds, a mixture including camphene, β-pinene, limonene and benzaldehyde induced chemotaxic behavior on the wasps and increased the parasitism on *E. heros* eggs, either in laboratory or semi-field test.

Introduction

Successful parasitism of egg parasitoids is due to their ability of recognize a variety of signals, including kairomones that play a major role in their foraging strategy for location and recognition eggs at long and short-range cues (Vet & Dicke, 1992; Turlings et al., 1993; Godfray, 1994; Vinson, 1998; Conti & Colazza, 2012). Moreover, parasitoids need to cope the high level of environmental chemical complexity and identify the right cues, which are embedded in a background of other compounds (Schröder & Hilker, 2008). Therefore, searching strategies are a complex process, especially for egg parasitoids, due to their small sizes and the inapparency of host eggs, thus direct signals may have low detectability, but high reliability (Vinson, 1994; Vet et al., 1995). Egg volatiles have been showed as an essential kairomone to guide many Scelionidae (Bin et al., 1993; Borges et al., 1999; Tognon et al., 2014; 2016), Trichogrammatidae (Renou et al., 1992; Bai et al., 2004; Yong et al., 2007) and Mymaridae (Conti et al., 1996).

Some clear cases showed Scelionidae being attracted by their host egg masses, crude extracts and/or synthetic compounds (Bin et al., 1993; Borges et al., 1999; Tognon et al., 2014; 2016). Bin et al. (1993) was the starting point to Heteroptera/Scelionidae egg volatiles interaction. They reported *Trissolcus basalis* being attracted by *Nezara viridula* egg extract and secretions from host female ovarioles.

For some *Telenomus* and/or *Trissolcus* species it was shown that kairomones are present in the adhesive secretion from the colleterial glands of Lepidoptera (Nordlund et al., 1987; De Santis et al., 2008) and follicular cells of heteropteran hosts (Bin et al., 1993; Borges et al., 1999; Conti et al., 2003). To true bugs, their chemical nature, defined previously as glycoconjugate complexes (Bin et al. 1993) has recently been clarified (Aquino, 2011; Michereff et al., 2016; Tognon et al., 2016).

Telenomus podisi Ashmead (Hymenoptera: Scelionidae) is known as one of the most important egg parasitoids of Pentatomidae with a close association for *Euschistus* spp, (Sujii et al., 2002; Tillman, 2011) and, in Brazil, *Euschistus heros* (Fabricius), a soybean pest, is its preferred host (Corrêa-Ferreira & Moscardi, 1995; Tognon et al., 2014). Thus, we aim to identify volatile substances on external surface of *E. heros* eggs and report their kairomonal activity to *T. podisi* under laboratory and semi-field conditions.

Materials and methods

We reared insects and carried out bioassays under controlled condition chamber (26 ± 1 °C, 65 ± 10% RH, 14h photophase), in the Ethology and Insect Chemical Ecology Laboratory, at Federal University of Rio Grande do Sul (UFRGS), Brazil.

Acquisition and rearing of insects

We stablished an *E. heros* colony from egg masses supplied by Genetic Resources and Biotechnology Laboratory (EMBRAPA/Cenargen) in Brasilia, DF, Brazil.

The adults were reared in 19 x 25 x 19 cm plastic cages, supplied with water in a glass shell vial with a cotton wick, and fed with fresh green beans, soybean, sunflower and peanut seeds as recommended by Borges et al. (2006).

Paper towel served as an oviposition substrate. Eggs were collected daily and kept under the same conditions in separate nymphal rearing cages or removed for use in bioassays. Newly emerged adults were removed daily from the immature cages and transferred to separate ones containing recently emerged adults. Each cage contained no more than 50 adults and the food was replaced twice weekly.

Telenomus podisi was also obtained from EMBRAPA/Cenargen. We continued to rear them in laboratory on the same host as provided, *E. heros*. Wasps were kept in glass tubes (7.5 x 1.3 cm) sealed with Parafilm® (Bemis Flexible Packaging, Neenah, WI) and fed with a drop of honey. In our experiments, only females, previously paired with males for 24 h, were used (approximately 48 h old). Each wasp was tested only once.

Preparation of egg extract

Mated *E. heros* females were separated from males and kept in different cages with food, water and organza material for oviposition. Eggs (12-24 h old) were removed from substrate (with forceps), weighed and placed in glass vials (4 mL clear vial, W/PTFE cap; Sigma-Aldrich St. Louis, Missouri, USA). Enough hexane (99%, Sigma-Aldrich) was added to ~1 g quantities of eggs to cover them, and after 5 min the solvent was transferred by syringe to another clean glass vial. The samples were kept at -4 ºC until use. Some of these samples were used in bioassays, the remainder (8) were analyzed at EMBRAPA/Cenargen by gas chromatography coupled with mass spectrometry (GC-MS).

Chemical identification

Egg extracts, previously concentrated to 50 µL under flux of N_2, were analyzed by GC (Agilent 7890A, DB-5MS) with a 30 m x 0.25 mm ID column and 0.25 µm film thickness, (J&W Scientific, Folsom, CA, USA), using a temperature program of 50 °C (2 min), 5 °C min^{-1} to 180 °C (0.1 min), and 10°C min^{-1} to 250 °C (20 min). To the analyses, 1 µL of (2)-ethyl hexanoate was added as an internal standard (IS) with a final concentration of 0.25 µg/mL. One microliter of each sample was injected using the splitless mode with helium as the carrier gas in a gas chromatograph with flame ionization (CG-FID Shimadzu 17A). Quantification of compounds was conducted by comparing the areas of each compound to the area of the internal standard used. Data were collected through Class-GC software.

For qualitative analysis of extracts a gas chromatograph was used (Agilent 5975 MSD) coupled to a mass selective detector (GC-MS) with ionization by electron impact (ionization energy 70 eV) and quadrupole analyzer. The temperature program injection mode, the column and the carrier gas were identical to those used in GC-FID. The fragmentation pattern of the compounds was compared to the data of mass spectrum library (NIST, Wiley-database, 2008). Identifications were confirmed by comparison of retention times and mass spectra with authentic standards obtained commercially (Sigma Aldrich® / Bedoukian®), as well as the calculation of Kovats indices.

Bioassays

Olfactometry

All olfactometer bioassays were conducted in an acclimatized room (24 ± 2°C and 60 ± 10% RH) during the photophase period and under fluorescent bulb (9 W, luminance = 290 lux).

The behavior of *T. podisi* females was observed in a two choices test using a horizontally positioned Y-tube olfactometer (1.4-cm diameter), with a 16-cm basal arm, bifurcated at 60° into two 19-cm arms. Airflow was 0.8 $L.min^{-1}$ provided by a vacuum pump connected to a flow meter and a humidifier. Before experiment, each female was placed individually into a glass tube (5 mL) and provided a drop of honey (3 µL) as food. A filter paper piece (1 x 2 cm, P5 Fisherbrand®, Fisher Scientific, Marshalltown, IA, USA) with synthetic compounds from eggs was tested.

A single wasp was introduced into the Y-tube and permitted to choose between the odor test (5 µL of synthetic compounds from eggs) or the same volume of hexane (control). Each insect was given 10 min to make a choice of arms in the olfactometer. Parasitoids that moved at least 3 cm into one branch arm and remained there for at least 60 s were recorded as responsive. If no choice was made in 10 min, the assay was concluded and the insect considered non-responsive, being excluded from statistical analysis.

The olfactometer was rotated 180° every three trials, washed after nine, with water and acetone and dried at 100 °C. After this procedure, the tissue papers with test substances were renewed.

The synthetic compounds were separated in three groups, according to their chemical profile and boiling point (terpenes plus aldehyde, ester and alkanes) For terpenes and aldehyde, the following mixtures were tested: A mixture (AM), composed by α-pinene (4ng/5μL), camphene (4ng/5μL), β-pinene (2ng/5μL), β-myrcene (2ng/5μL), limonene (12ng/5μL) and benzaldehyde (3ng/5μL); B (BM) [AM removed α-pinene]; C (CM) [AM removed α-pinene and limonene]; D (DM) [AM removed α-pinene and camphene]; E (EM) [AM removed α-pinene and benzaldehyde]; F (FM) [AM removed α-pinene and β-pinene]; G (GM) [AM removed α-pinene and β-myrcene]; H (HM) [AM removed α-pinene, β-myrcene and limonene]; I (IM) [AM removed α-pinene, β-myrcene and camphene]; J (JM) [AM removed α-pinene, β-myrcene and benzaldehyde]; K (KM) [AM removed α-pinene, β-myrcene and β-pinene]. To ester group, we tested *Z*-9 tetradecenyl acetate (16 ng/5μL). To the third group, we evaluated the alkanes in two mixtures, the A mixture (AAM) composed by C_{12}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18} and C_{19} (2ng/5μL); and in the B mixture (ABM) C_{20}, C_{21} (2ng/5μL), C_{23}, C_{24} (3ng/5μL), C_{25} e C_{26} (9ng/5μL). The solvent hexane (H) was the control treatment. We carried out at least 40 replicates to each treatment.

Parasitism tests

No-choice laboratory parasitism tests were performed with *T. podisi* females (48 h old). They were individually kept into a glass tube (7.5 x 1.3 cm), with a drop of honey, sealed with Parafilm® (Bemis Flexible Packaging, Neenah, WI) and offered 10 *E. heros* eggs (glued over a filter paper piece and with a double side tape) either coated with 5 μL of hexane (control) or 5 µL of synthetic G mixture (limonene, camphene, benzaldehyde and β-pinene), an attractive with less substances. After 3 hours, the females were removed from the glass tubes and the egg masses observed daily to report parasitism or nymphal emergence. We carried out, at least, 30 replicates/treatment.

The G mixture was also evaluated under semi-field conditions. These experiments were executed in a cage (90 x 90 x 200 cm) in an open area (27 ± 2°C, 72 ± 20% RH) at Agronomy School (30°05′27″ S, 51°40′18″ W) in Porto Alegre, Rio Grande do Sul. The cage contained 12 soybean plants (grown crop TEC 5936 IPRO) on the reproductive phase R4-R5, into plastic black containers (8 L). *Euschistus heros* eggs (n= 20, 24 h old) were glued on paper with double-sided tape (Scotch®) to a wooden support (30 cm) inside the pots, in four plants. On the top of two egg masses we added 5 µl of synthetic GM and the same volume of hexane (control) on the other two. After that, we released into the cage 30 mated *T. podisi*, which were exposed to eggs for 6 h, being removed and placed in glass tubes, as previously described, after that. Emergence or eclosion were checked daily. We performed 34 replicates.

Parasitoids were sent to Dr. Valmir Antônio Costa from Biological Institute of São Paulo, Brazil for confirmation; voucher specimens are deposited in the collection of Biological Institute.

Statistical analyses

The data from individual compounds did not followed a normal distribution, thus the differences of the amounts of each individual compound were individually compared by nonparametric statistics using Kruskal-Wallis test and submitted to Dunn test with 95% confidence. Data from parasitism’s bioassays were, as well, compared with the same test.

First choice in olfactometer and differences in the proportion of *T. podisi* females choosing a particular odor source were analyzed by Chi-square test. All analyses were performed in Bioestat® 5.0 software ($P < 0.05$) (Ayres et al., 2007).

Results

Chemical identification

Thirty two compounds were identified from *E. heros* egg extracts which are include in the following chemical groups: terpenes (α-pinene, camphene, β-pinene, β-myrcene and limonene), alkanes (C_{11} to C_{31}), aldehyde (benzaldehyde) and ester (*Z*-9 tetradecenyl acetate). Among terpenes, α-pinene was the minority ($P < 0.05$), the others were not significant different on quantity ($P > 0.05$). To alkanes, C_{28} was the majority compound ($P < 0.05$) (Table 1).

Olfactometry

Telenomus podisi females did not show a significant choice between the synthetic AM (α-pinene + camphene + β-pinene + β-myrcene + limonene + benzaldehyde) (40.47%) and hexane (50%) ($\chi^2 = 0.842$; fd = 1; $P = 0.4913$). On the other hand, when α-pinene was removed from the mixture (BM) the wasps showed a positive response (52.38%) rather than hexane (26.19%) ($\chi^2 = 7.333$; fd = 1; $P = 0.0138$). A similar result we observed without α-pinene and β-myrcene from AM (GM) ($P < 0.05$). However, the choice between CM, DM, EM, FM, HM, IM, JM and LM was not different when compared to the control ($P > 0.05$) (Figure 1). *Telenomus podisi* chemotactic responses to *Z*-9 tetradecenyl acetate

(50%) was not significant different to hexane (40.47%) ($\chi^2 = 0.842$; fd = 1; P = 0.4913). Similar results to alkanes (AAM and ABM) versus control were also registered (P > 0.05).

Parasitism tests

We observed a greater parasitism on *E. heros* egg masses with GM (52.42%) than with hexane only (33.93%) in laboratory test (H = 4.9547; fd = 1; P < 0.026). Similar result was reported under semi-field conditions (GM – 56.32% and control – 38.82%) (H= 7.2467; fd= 1; P < 0.0071). In both conditions, emergence was greater on GM than control (P < 0.05) and consequently, the nymph emergence was greater to control than GM (P < 0.05) (Table 2). Nymph emergence from egg masses not exposed to *T. podisi* was 98.23%.

Discussion

We presented a variety of compounds found on the external layer of *E. heros*, including different chemical groups, i.e., terpenes, alkanes, an aldehyde and a ester. Substances from *E. heros* eggs was first observed by Aquino (2011), however it was identified only compounds derived from long chain fatty acids. Lately, Michereff et al. (2016) also found compounds with similar profile in the same species. Nevertheless, except for limonene cited in the last work, none was registered in the present study. The difference between these results might be associated to polar solvents used by them, which allowed substances extraction in the indoor liquid egg, different from the nonpolar solvent (hexane) applied by us, that extracts manly substances on external layer of egg chorion, as observed by Conti el al. (2003).

Although many compounds have been identified in eggs, only the mixture of some terpenes and aldehyde triggered the chemotactic response in *T. podisi* females. Volatiles such as limonene and α-pinene are known as substances from plant secondary metabolism and also as insect repellents (Nerio et al., 2010). Therefore, we observed that blends without α-pinene (BM) and those without β-myrcene plus α-pinene (GM) were attractive to wasps. It was already observed that semiochemicals on insect eggs may have a functional protection against natural enemies (Hinton, 1981; Blum, 1981; Blum & Hilker, 2002; Tognon et al., 2016), as we noticed to α-pinene and β-myrcene plus α-pinene. Nevertheless, some parasitoid can break up the external chemical barrier, using some of these cues as kairomones to find their target (Papaj & Lewis, 1993; Quicke, 1997; Sasaki & Godfray, 1999; Steidle & van Loon, 2002), even it does not seem to be true in the chemical interaction between *T. podisi* and *E. heros* eggs. We firstly observed that this species is attracted by egg masses (Tognon et al., 2014) and egg extract (R. Tognon unpublished yet) of *E. heros* in olfactometer system. Thus, we believe that α-pinene and β-myrcene naturally are mitigated on eggs by all the other substances present on there. Therefore, these terpenes may act as repellent for *T. podisi* only when they are withdrawn from the natural extract, as observed in our synthetic mixtures.

The *Z*-9 tetradecenyl acetate was present on *E. heros* but did not induced any response in *T. podisi*. Bai et al. (2004) reported (*E*)-12- tetradecenyl acetate, a compound of many sex pheromone of moths, from *Ostrinia furcanalis* (Guenee) (Lepidoptera: Pyralidae) eggs as a great kairomonal agent to *Trichogramma ostriniae* Pang et Chen (Hymenoptera: Trichogrammatidae). Thus, we hypothesize that this ester identified on *E. heros* eggs did not induced any response on *T. podisi* because it is not a relevant substance on ecological environment of this parasitoid. Moreover, perception of volatile kairomones is mediated by olfactory receptor neurons (ORNs), located primarily in the insect antenna, which act to convert the chemical signal into an electrical signal that inputs directly to the central nervous system (CNS) (Hansson 2002). The either positive or negative response for specific compounds or mixtures observed here can be related to odorant binding proteins (OBPs), some highly specific structures on the perception of molecules into antennal sensillum lymph. OBPs are a class of water-soluble proteins, which were discovered in several insect orders for liganding and degrading enzymes, however, the absence of response to *Z*-9 tetradecenyl acetate can be related to the absence of proteins to recognize the odor on *T. podisi* antenna (Vogt et al., 1999; McKenna et al., 1994, Dickens et al., 1995). However, further research should be conducted for better understand the ecological context of this substance on *E. heros* egg masses.

Regarding to alkanes we got C_{28} as the majority one, thus for having longer chains and low volatility, generally they work on host recognition and not location (Rutledge, 1996). Cuticle hydrocarbons are reported in great quantity on the insect exoskeleton (Gibbs, 1998; Howard & Blomquist, 2005). Colazza et al. (2007) identified carbons from C_{19} to C_{34} on the body of *Nezara viridula* L. (Hemiptera: Pentatomidae) adults, for providing intraspecific signals and/or interspecific cues that modify the behaviors of receiving organisms. The same substances were described on scales (Boo & Yang, 2000) and eggs of *Ostrinia nubilalis* (Hübner) (Lepidoptera: Crambidae) and *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Renou et al., 1992), in both studies they were cited as contact kairomone. Thus, we may suggest the absence of response to alkanes by *T. podisi* should be associated to their low volatility; they would act in a very short-range distance or only by contact. Furthermore, the G mixture was efficiency to increase the parasitism on *E. heros* eggs by *T. podisi* either on laboratory or semi-field condition. Our recent result asserts the later reported by Tognon et al. (2014) studying volatiles from egg masses, reporting a high attraction by *T. podisi* to *E. heros* eggs, Thus, we can claim that this parasitoid species use volatiles cues from *E. heros* external egg substances, as a short-range kairomone for location due to its greater volatility in relation to the internal ones reported by other authors. Semiochemicals related to location, recognition and acceptance could be a practical solution to attract and retain natural enemies in field areas where there are pest spots (Borges & Aldrich, 1994). One of the first studies applying semiochemicals on field was conducted by Lewis et al. (1975ab). They showed that application of an extract from *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae) scales or a synthetic kairomone onto soybean plants increased host egg parasitism by *Trichogramma* species, but only at high host densities. In Pentatomidae, a tentative was done using (*E*)-2- hexenal, a compound present in the metathoracic glands of *E. heros* which increased parasitoids population and parasitism of their host eggs laid naturally in treated areas of soybean field in a week-long experiment (Peres, 2004). One the other hand, a full crop season showed that the abundance of parasitoids did not differ from the control plots, however the parasitoids recruitment started at an early phenology stage of crop, i.e. before the host invaded and dominated the area (Vieira et al., 2014). A recent research using semiochemical from Pentatomidae under field conditions was made by Tognon et al. (2016) whose showed that egg parasitoids were attracted to the main compound present on *E. conspersus* egg masses, methyl (2*E*,4*Z*)-2,4-decadienoate, known as the principal substance of male-produced aggregation pheromone, which act only as kairomone when available in a very low concentration (1mg and 0.1 mg total).

Our research report relevant results to understand the interactions between Scelionidae/Pentatomidae, through the egg masses chemical identification and kairomonal activity on *T. podisi*. Based in our findings we can infer that *E. heros* has different and specific substance on eggs when compared to other insect species, while some of them has pheromonal components as observed in *E. conspersus* by Tognon et al. (2016), *E. heros* does not have this kind of elements as here reported. Repellent components acting as allomones to parasitoids seems to be frequently for pentatomid eggs. As a second point, we observed that chemicals on external surface of this studied stink bug are not the same from those into the internal embryonic nutritional liquid as reported by others authors, and then we infer that substances on external layer of eggs are strongly used by parasitoids as kairomones at short-range. Finally, we believe the understanding of these factors will help to improve the use of semiochemicals mainly on biological conservative programs as a purpose to attract and retain natural enemies in spot field where there is the pest.

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Figure caption

Figure 1. Response of *Telenomus podisi* (±SE) tested in olfactometer double choice to the following mixtures versus hexane: AM) limonene, camphene, benzaldehyde, β-myrceno, β-pinene and α-pinene; BM) limonene, camphene, benzaldehyde, β-myrcene and β-pinene; CM) camphene, benzaldehyde, β-myrcene and β-pinene; DM) limonene, benzaldehyde, β-myrcene and β-pinene; EM) limonene, camphene, β-myrcene and β-pinene; FM) limonene, camphene, benzaldehyde and β-myrcene; GM) limonene, camphene, benzaldehyde and β-pinene; HM) camphene, benzaldehyde and β-pinene; IM) limonene, benzaldehyde and β-pinene; JM) limonene, camphene and β-pinene; LM) limonene, camphene and benzaldehyde. Numbers represent the number of responsive insects. Bars followed by different letters within each treatment indicate difference (χ^2, $P < 0.05$).

Fig 1

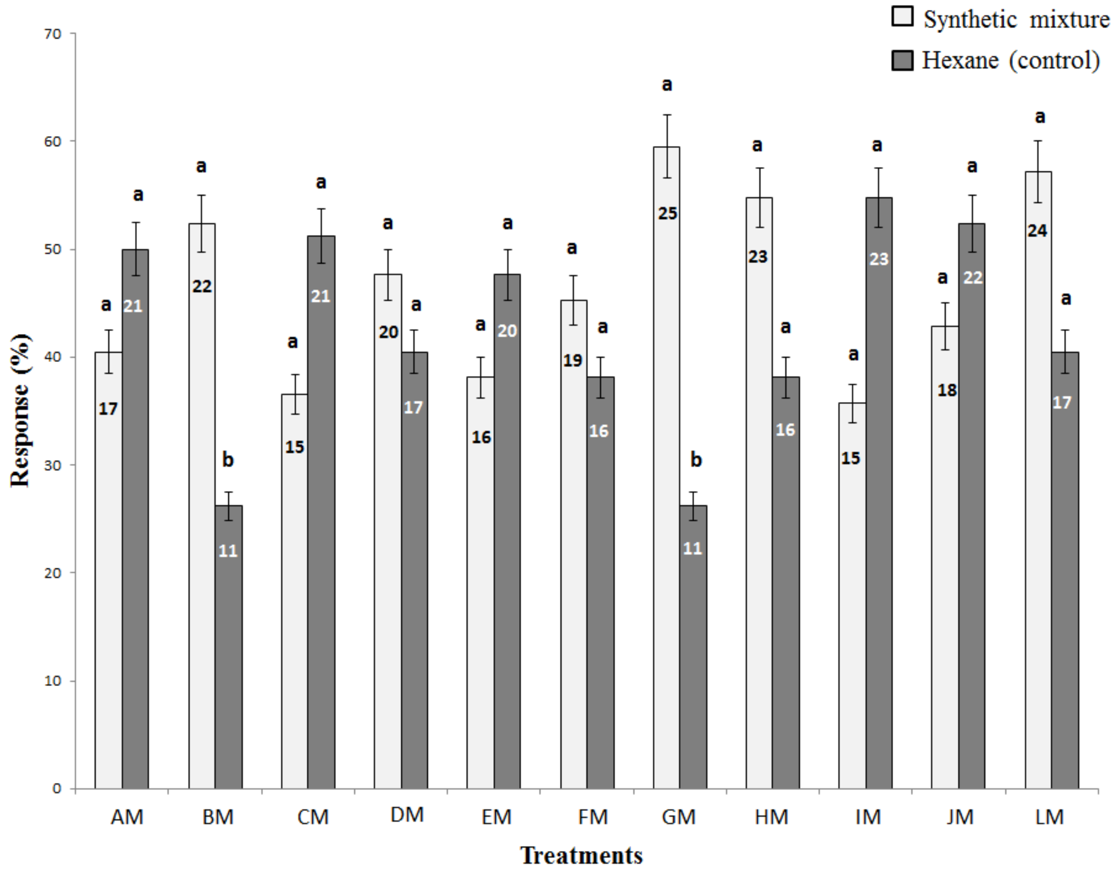

Table 1 Mean (ng/egg) ± (SE) of compounds extracted from 1 gram of *Euschistus heros* and retention index (RI) of compounds/chemical group identified on *E. heros* eggs

Group/compound	**Mean ng/egg (±SE)**	**RI (DB-5MS)**
Terpenes		
α-pinene	0.07±0.064 a*	933
Camphene	3.86±2.947 b	949
β-pinene	2.91±1.448 b	977
β-myrcene	2.14±0.810 b	988
Limonene	1.63±0.343 b	1029
Alkanes		
Undecane (C_{11})	1.77±0.374 b	1100
Dodecane (C_{12})	1.69±0.218 b	1200
Tridecane (C_{13})	3.79±0.905 b	1300
Tetracosane (C_{14})	1.77±0.267 b	1400
Pentacosane (C_{15})	1.23±0.310 b	1500
Hexadecane (C_{16})	1,01±0,268 b	1600
Heptadecane (C_{17})	1.49±0.403 b	1700
Octadecane (C_{18})	4.53±1.943 b	1800
Nonadecane (C_{19})	2.46±0.649 b	1900
Eicosane (C_{20})	1.42±0.401 b	2000
Heneicosane (C_{21})	1.30±0.701 b	2100
Docosane (C_{22})	1.97±1.043 b	2200
Tricosane (C_{23})	3.40±1.088 b	2300
3-Methyl tricosane	0.09±0.081 b	2367
Tetracosane (C_{24})	3.15±0.883 b	2400
Z-12-Pentacosane	21.86±4.327 c	2476
Pentacosane (C_{25})	10.49±4.840 b	2500
3-ethyl tetracosane	0.11±0.069 b	2565
Hexacosane (C_{26})	8.55±2.272 c	2600
Heptacosane (C_{27})	26.72±9.642 c	2700
13-Methyl heptacosane	7.78±5.595 b	2733
Octacosane (C_{28})	73.52±61.530 a	2800
Nonacosane (C_{29})	0.89±0.815 b	2900
Triacontane (C_{30})	0.42±0.387 b	3000
Hentriacontane (C_{31})	8.02±4.651 b	3100
Aldehyde		
Benzaldehyde	4.13±1.922	958
Ester		
Z-9 tetradecenyl acetate	11.81±6.063	1794

* Means followed by same letter in the same functional group does not differ by Kruskal-Wallis (*P* > 0.05)

Table 2 Parasitism of *Euschistus heros* eggs with synthetic G mixture (GM) or hexane (H) under laboratory or semi-field condition by *Telenomus podisi*

Condition	Treatments	No. exposed eggs	% Parasitized eggs*	% Parasitoid emergence*	% Nymphal emergence*	% Egg mortality [a]
Laboratory	GM	330	52.42^{a}	43.03a	45.45^{a}	2.12
	H	330	33.93b	28.78b	66.06b	0.9
Semi-field	GM	680	56.32a	54.26a	42.64a	2.35
	H	680	38.82b	38.82b	58.97b	1.47

*Numbers followed by different letters are significantly different within each treatment/condition/column at Kruskal-Wallis, $P < 0.05$

[a] These include the percentage of eggs from which neither parasitoids nor *E. heros* nymphs emerged

5 ARTIGO 3

Volatiles mediating parasitism of *Euschistus conspersus* and *Halyomorpha halys* egg by *Telenomus podisi* and *Trissolcus erugatus**

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Volatiles Mediating Parasitism of *Euschistus conspersus* and *Halyomorpha halys* Eggs by *Telenomus podisi* and *Trissolcus erugatus*

Roberta Tognon[1], Josué Sant'Ana[1], Qing-He Zhang[2], Jocelyn G. Millar[3],

Jeffrey R. Aldrich,[4,5]* Frank G. Zalom[5]

[1] PPG-Fitotecnia, Faculdade de Agronomia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil

[2] Sterling International, Inc., Spokane, WA 99216, USA

[3] Department of Entomology, University of California, Riverside, CA 92521, USA

*[4] Jeffrey R. Aldrich Consulting LLC, Santa Cruz, CA 95061, USA; * drjeffaldrich@gmail.com; Phone: (301)503-8288*

[5] Department of Entomology and Nematology, University of California, Davis, CA 95616, USA

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Abstract— This study identified chemical compounds found on the eggs of two stink bug species, one native to western North America, *Euschistus conspersus*, and an invasive species from Asia, *Halyomorpha halys.* The responses of two scelionid egg parasitoids, *Trissolcus erugatus* and *Telenomus podisi*, toward the natural stink bug egg volatiles, and synthetic reconstructions of the egg volatiles, were tested in bioassays. A compound previously identified as the major component of the male-produced aggregation pheromone of *E. conspersus*, methyl (2*E*,4*Z*)-2,4-decadienoate, was the major volatile identified from extracts of *E. conspersus* eggs. In contrast, for *H. halys*, the sesquiterpenoid compounds that comprise the male-produced aggregation pheromone of this species were not detected on their eggs; however, hexadecanal, octadecanal, and eicosanal were identified in extracts of *H. halys* eggs. In laboratory olfactometer tests, both *Tr. erugatus* and *Te. podisi* females were attracted to extracts of *E. conspersus* eggs, and to synthetic methyl (2*E*,4*Z*)-2,4-decadienoate. However, female *Tr. erugatus* and *Te. podisi* wasps were repelled both by extracts of *H. halys* eggs, and by a blend of the aldehydes identified from *H. halys* eggs. A follow-up field study, using hexane-washed and intact *E. conspersus* as sentinel eggs, showed that the parasitoids *Trissolcus erugatus* and *Gryon obesum* emerged from these eggs. Sentinel hexane-washed eggs treated with 3 ng of methyl (2E,4Z)-2,4-decadienoate were parasitized more by these two species than were hexane-washed or unwashed eggs, whereas hexane-washed eggs treated with a comparable dose of the C16,18,20 aldehyde mixture were avoided by these parasitoids. In a further field experiment, *Trissolcus basalis* was the primary parasitoid found in sticky traps baited with methyl (2E,4Z)-2,4-decadienoate, indicating that this species was attracted to, but either did not oviposit or develop in the *E. conspersus* sentinel eggs in the previous experiment.

Key Words— Egg parasitoids, Kairomones, Biological control, Octadecanal, Methyl (2*E*,4*Z*)-2,4-decadienoate, Hopkins' host-selection principle, Heteroptera, Pentatomidae, Scelionidae

Introduction

Complexes of stink bug species (Heteroptera: Pentatomidae) attack a multitude of agricultural crops worldwide, including cotton, corn, and soybeans (e.g., Tillman 2011) and, in California, tomatoes (Zalom et al. 1997). Invasive stink bug species that have established in California, such as the cosmopolitan pest *Nezara viridula* (L.) (Hoffmann et al. 1991), the crucifer specialist *Bagrada hilaris* (Burmeister) (Palumbo et al. 2016), and, most recently, the polyphagous brown marmorated stink bug, *Halyomorpha halys* (Stål) (Hoebeke and Carter 2003), are increasing in importance as pests of Californian crops. Stink bug populations are suppressed by a variety of generalist predators (e.g., Krupke and Brunner 2003; Tillman et al. 2015), but egg parasitoids likely provide the most effective biological control of stink bugs because of their ability to kill potential pentatomid pests before plant damage occurs (Conti and Colazza 2012; Sithanantham et al. 2013). Egg parasitoids are, in fact, the largest group of entomophagous insects associated with Heteroptera (Conti and Colazza 2012). In California, introduction of the scelionid egg parasitoid, *Trissolcus basalis* (Wollaston), from France, Italy, and Spain has been largely responsible for the decline of *N. viridula* to below economic threshold levels (Hoffmann et al. 1991).

Of the complex of native stink bugs that feed on tomatoes in California, the consperse stink bug, *Euschistus conspersus* Uhler (Heteroptera: Pentatomidae), is the most important (Cullen and Zalom 2005). Nymphs and adults of *E. conspersus* severely damage green and mature red fruit by injecting salivary enzymes as they feed (Zalom et al. 1997). *Halyomorpha halys* looms as a potentially damaging stink bug invader in California because of its wide host plant range (Joseph et al. 2015), and because its population and distribution are still expanding (StopBMSB.org 2015). In addition, brown marmorated stink bug adults are a nuisance due to their propensity to overwinter in buildings (Cira et al. 2016), sometimes in large numbers (Inkley 2012). In its native range in Asia, H. halys is an occasional or outbreak pest of numerous crops (Lee et al. 2013), but in the U.S., it has caused severe economic losses in the mid-Atlantic states on peaches, pears, apples, grapes, soybeans, sweet corn, green beans, eggplant, tomatoes, and other crops (Joseph et al. 2015; Rice et al. 2014). Moreover, *H. halys* is established and spreading in Canada (Fogain and Graff 2011) and Europe (Gariepy et al. 2015; Haye et al.

2015; Wermelinger et al. 2008), and is threatening to become a global pest (Zhu et al. 2012).

Parasitoid wasps in the subfamily Telenominae (Hymenoptera: Scelionidae) (Talamas and Buffington 2015) develop inside eggs of other insects, mainly those of Heteroptera, Lepidoptera, Diptera, and Neuroptera (Taekul et al. 2014). Based on molecular data, it appears that stink bugs (Pentatomidae) and seed bugs (Lygaeidae) are the ancestral hosts for the two telenomine core genera, *Telenomus* and *Trissolcus* (Taekul et al. 2014). *Telenomus podisi* Ashmead is a cosmopolitan parasitoid of eggs of many pentatomid species (e.g., Tillman 2010), particularly *Euschistus* eggs (Hoffmann et al. 1991; Tillman et al. 2010). In its native range, the most important egg parasitoids of *H. halys* are *Trissolcus* species (Lee et al. 2013; Rice et al. 2014). Although some North American and European *Trissolcus* wasps found fresh sentinel *H. halys* eggs in wooded field sites, few were able to develop successfully in these fresh *H. halys* eggs (Haye et al. 2015; Herlihy et al. 2016; Talamas et al. 2015a). Therefore, explorations for parasitoids in the Asian range of *H. halys* were undertaken, with two *Trissolcus* species from China, *Tr. japonicus* (Ashmead) and *Tr. cultratus* (Mayr), being identified as promising candidates for classical biological control of *H. halys* in regions where it has been introduced (Haye et al. 2015; Talamas et al. 2015a). Surprisingly, *Tr. Japonicus* recently has been found established in both the eastern and western U.S., presumably by adventitious introductions (Herlihy et al. 2016; Lara et al. 2016; Talamas et al. 2015a).

Besides pupae, insect eggs are the most inconspicuous host stage attacked by parasitic wasps, yet chemicals directly associated with a host’s eggs are the ultimate cues triggering successful oviposition by egg parasitoids (Fatouros et al. 2008). This incongruence confronting foraging egg parasitoids is an example of the ‘reliability-detectability dilemma’ described by Vet and Dicke (1992) in their seminal effort to organize literature on insect parasitism. Potential foraging cues, such as host-plant volatiles, may be highly detectable at long distances but may not reliably predict the presence of hosts, whereas chemicals on or in host eggs are the most reliable cues for host recognition, but these compounds may be present in such low concentrations as to challenge detection by foraging parasitoids. This type of selection on eggs favors inconspicuousness.

In their review, Conti and Colazza (2012) followed the conceptual approach of Vet and Dicke (1992) for the chemical ecology of egg parasitoids associated with true bugs. Foraging *Telenomus* and *Trissolcus* females exemplify this reliability/detectability spectrum enabling them to find their hosts quickly. In various species, this spectrum ranges from: 1) attraction of female wasps to male-produced aggregation pheromones of their host, followed by phoresy on the mated host females (Aldrich et al. 1984; Bruni et al. 2000); 2) attraction to maleproduced pheromones (Borges et al. 1999; Krupke and Brunner 2003; Tognon et al. 2014) or to defensive secretions of adults (Laumann et al. 2009; Mattiacci et al. 1993) to arrive in the vicinity of potential host eggs; 3) attraction to plant volatiles induced by the combined feeding and oviposition by adult host females (Colazza et al. 2004); 4) intensified searching in areas where gravid females of potential hosts have left chemical 'footprints' (Colazza et al. 2007; Salerno et al. 2009); and 5) recognition of odors from adhesive secretions used to attach eggs to each other and the substrate (Bin et al. 1993; Borges et al. 1999).

Earlier research by Tognon et al. (2014) on the generalist pentatomid egg parasitoid *Te. podisi* from Brazil showed that female wasps from a colony maintained for many generations on eggs of the stink bug *Euschistus heros* (Fabricius) preferred to oviposit in eggs of *E. heros* rather than in eggs of the rice stink bug, *Tibraca limbativentris* Stål, whereas female wasps from *T. limbativentris* eggs collected in a rice field, preferred to oviposit in *E. heros* eggs after being reared for just one generation on the latter host. In contrast, *Te. podisi* reared directly from rice stink bug eggs did not exhibit a preference for eggs of *T. limbativentris* over those of *E. heros*. The present study is an extension of this earlier research on scelionid/pentatomid egg parasitism (Tognon et al. 2014) using eggs of *E. conspersus* and *H. halys*, and the native North American egg parasitoids *Te. podisi* and *Trissolcus erugatus* Johnson. The primary objective of this study was to identify the volatiles from the stink bug eggs, and test egg extracts and individual compounds and blends of compounds identified from the egg extracts, in both laboratory and field bioassays. Our results are similar to those of Tognon et al. (2014), and provide a chemical explanation for the divergent responses of scelionid parasitoids toward the eggs of different stink bug species, thus highlighting a little known level of chemical communication in Heteroptera.

Methods and Materials

Stink Bug Colonies A colony of *E. conspersus* nymphs and adults was obtained from Paramount Farming in Shafter, Kern County, CA, USA in December 2014 from collections made in the southern San Joaquin Valley during the summer of 2014. This colony was maintained in a Percival Model I-36LL growth chamber (Perry, IA, USA; 26 ± 1 °C, 65 ± 10 % RH, 16 L:8D photoperiod) at the University of California at Davis (UCD). Adults were maintained in 19 × 25 × 19-cm cylindrical plastic containers (accumulated from Trader Joe's grocery, Monrovia, CA, USA) with a 13 × 13-cm hole in the top fitted with organza material. Insects were fed organic green beans, sunflower seeds, and cherry tomatoes, with water provided from cotton-stoppered polypropylene shell vials (4.0 ml, 15mmdiam, 45mmheight; J. G Finneran Associates, Inc.,Vineland, NJ, USA). Each cage contained no more than 50 adults, and the food was replaced twice a week. Paper towel-lined cages served as an oviposition substrate. Eggs were collected from the cages daily, and maintained separately under the same conditions as above for colony maintenance, and used in bioassays.

Halyomorpha halys nymphs and adults were collected in 2015 from Fremont Community Garden in Sacramento County, CA, USA. A colony of *H. halys* was maintained as described above for *E. conspersus*, except that pumpkin seeds and slices of apple, plum or cherries also were provided.

A colony of the spined soldier bug, *Podisus maculiventris* Say (Heteroptera: Pentatomidae), was established from adults collected at the arboretum of the University of California at Santa Cruz (UCSC) in Rescue® Stink Bug Traps (Sterling International, Inc., Spokane, WA, USA) baited with the synthetic aggregation pheromone of *P. maculiventris* (Aldrich et al. 1984). The *P. maculiventris* colony was maintained as for the other stink bugs, except that insects were fed larvae and pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) obtained commercially (Rainbow Mealworms, Compton, CA, USA).

Parasitoid Colonies *Trissolcus erugatus* and *Te. podisi* were collected in the Student Farm at UCD and the UCSC arboretum using fresh sentinel egg masses of *E. conspersus* and *P. maculiventris*, respectively. *Trissolcus erugatus* was identified using the key of Talamas et al. (2015b); *Te. podisi* was identified using the key of Johnson (1984). Voucher specimens for each parasitoid species have been deposited in the UCD R. M. Bohart Museum of Entomology. Eggs were collected daily, and exposed in the field the same day by clipping masses onto leaves of garden plants for *Tr. erugatus* or onto coast live oaks (Quercus agrifolia) for *Te. podisi*. After 48 h, eggs were removed from the field, and held for possible parasitoid emergence. Both species were maintained in a Percival Model I-36LL growth chamber at 26 ± 1 °C, 65 ± 10 % RH, 14 L:10D photoperiod, using *E. conspersus* eggs; adult wasps were fed honey only.

Preparation of Egg Extracts To collect eggs from *E. conspersus* and *H. halys*, mated females were separated from males and kept in different cages with food, water, and paper toweling. Eggs (12–24-h-old) were removed from the paper with forceps, while wearing unpowdered latex gloves, placed in glass vials (2 ml borosilicate; Waters Corp., Milford, MA, USA), and weighed. Enough tert-butyl methyl ether (99.8 %; Sigma-Aldrich, St. Louis, MO, USA) to cover ~1 g of eggs was added; after 5 min, the solvent was transferred by syringe to another clean glass vial. Six samples of egg extracts from each species were analyzed. Other samples used for laboratory bioassays were prepared similarly, but using hexane as the solvent (>99 %, Sigma-Aldrich); samples were kept at −4 °C until use. Tert-Butyl methyl ether was the solvent of choice for egg extracts for chemical analysis because of its greater purity in the range of early eluting volatiles, while hexane was used for egg extracts for bioassays because, unlike tert-butyl methyl ether, it lacks a strong odor.

Dissection of Male Stink Bugs The genital capsules (terminal segment) of wild *E. conspersus* and *H. halys* males were dissected and extracted as follows. Soon after being captured in the field, adult males were killed by freezing at −4 °C for 20 min, and the genital capsule was clipped from the terminus of each male under a stereomicroscope using clean forceps and micro-scissors. Four or five capsules were clipped into 2 ml glass vials, and covered with tert-butyl methyl ether. After 5 min, the solvent was transferred by syringe to another clean glass vial. Five samples for each species were analyzed.

Chemical Identifications The stink bug egg extracts or male genital capsule extracts were concentrated to 20 µl under N_2, and analyzed by coupled gas chromatography-mass spectrometry (GC-MS) using an HP 6890 GC in series with an HP 5973 mass selective detector in the electron impact mode at 70 eV using an HP-5MS GC column (30 m × 0.25 mm × 0.25 µm film thickness; J & W Scientific, Folsom, CA, USA). Three µl of each concentrated sample was injected into the GC-MS in splitless mode. Helium was the carrier gas, and the injector and detector temperatures were 250 and 300°C, respectively. Column temperature was programmed from 50°C for 1 min, to 240°C at 10°C/min, with a final hold for 10 min. Compounds of interest were tentatively identified using Wiley7N (Scientific Instrument Services, Inc., Ringoes, NJ, USA) and PAL 600K (Palisade Corp., Ithaca, NY, USA) spectral libraries. Key compounds were fully identified by comparison of retention times and mass spectra to those of authentic standards. Fifty ng of octyl acetate (>99% Sigma-Aldrich) was added to some unconcentrated samples before GC-MS analysis as an internal standard (IS) for compound quantification. The standard of the main pheromone component of *Euschistus conspersus* (Aldrich et al. 1991), methyl (2*E*,4*Z*)-2,4-decadienoate was purchased commercially (>90%; Bedoukian Research, Inc., Danbury, CT, USA). For identification of the aldehydes found on the eggs of *H. halys*, a crude mixture of C_{14-20} aldehydes was prepared by oxidation of the corresponding commercially available primary alcohols as described in detail below; $C_{14,15,17}$:OH (97%, 97%, 98%, respectively; Sigma-Aldrich), $C_{16,19}$:OH (97% and 98%, respectively; TCI America, Portland, OR, USA), C_{18}:OH (97%; Avocado Research Chemicals, Haysham, UK), and C_{20}:OH (96%; Lancaster Synthesis, Pelham, NH, USA). For laboratory olfactometer studies and field experiments, a purified synthetic mixture of $C_{16,18,20}$ aldehydes (500 ng/µl hexane) was prepared at UC Riverside as follows. The $C_{16,18,20}$ straight-chain aldehydes appeared to be present in a ratio of ~4.5:4.5:1 in extracts of *H. halys* eggs (see below), so the aldehydes were synthesized as a blend, rather than making the individual compounds. Thus, hexadecanol (0.55 g, 2.25 mmol), octadecanol (0.61 g, 2.25 mmol), and eicosanol (0.15 g, 0.5 mmol) were dissolved in dry methylene chloride and the solution was cooled to 0ºC. A mixture of pyridinium dichromate (2.65 g, 7 mmol) and 2.5 g powdered 4Å molecular sieve were ground together in a mortar and pestle, and then added in one portion to the solution of alcohols. The cooling bath was removed and the mixture was warmed to room temperature and stirred for 2 h, then the mixture was diluted with 100 ml of hexane and stirred 15 min. The resulting slurry was filtered through a celite pad. The filtrate was concentrated by rotary evaporation, then taken up in 10 ml of hexane and purified by vacuum flash chromatography on 40 g silica gel pre-wetted with hexane, eluting with 1 x 50 ml of hexane, and 6 x 50 ml of 5% EtOAc in hexane. Fraction 4 containing the aldehydes was concentrated (~75% overall yield), transferred to an ampoule, made up to 10 ml with hexane, and a small crystal of butylated hydroxytoluene was added as a stabilizer. The ampoule was sealed and shipped by overnight courier to UCD for bioassays. A subsample of the aldehyde mixture was sent to the Spokane laboratory for GC-MS analysis under the same conditions as for the natural product samples.

Laboratory Bioassays The behavior of *Te. podisi* females reared from *E. conspersus* eggs was observed in a two-choice test using a horizontally positioned Y-tube olfactometer (1.4-cm diameter), with a 16 cm basal arm, that bifurcated at a 60° angle into two 19-cm arms. Airflow was 0.8 l/min provided by a vacuum pump connected to a flow meter and a humidifier. Before the experiment, each female was placed individually in a glass tube (5 ml), and provided with a drop of honey. The Y-olfactometer was surrounded by a paper wall to minimize possible cues from the room, and was illuminated by a white compact fluorescent bulb (9W) located above the device. The temperature in the bioassay room was maintained at 22°C. A piece of filter paper (1 x 2 cm, P5 Fisherbrand®, Fisher Scientific, Marshalltown, IA, USA) with 5 µl of a hexane solution of the test substance was placed in one arm of the olfactometer; the other arm contained tissue paper with 5 µl of hexane. A single wasp was introduced into the Y-tube, and allowed to choose between the arm with the egg extracts of *E. conspersus* (10 egg equivalents; EE) or the hexane control arm. The same procedure was followed for egg extracts of *H. halys* (4.5 EE), and the synthetic compounds identified in egg extracts were tested similarly. The main pheromone component of *E. conspersus* males (methyl (2*E*,4*Z*)-2,4-decadienoate) was tested at two different concentrations (0.2 and 2 ng/µl hexane, or 1 and 10 ng total), and the mixture of

$C_{16,18,20}$ aldehydes was tested at 0.02 ng/µl hexane (0.1 ng total); hexane alone was the control for all bioassays.

Each wasp was allowed 10 min to choose one of the olfactometer arms, and then was discarded whether or not it had made a choice. A choice was defined as when a wasp passed a line 3 cm into either arm of the Y-tube, and remained there for 1 min. Unresponsive females were those that did not move during the first 5 min or did not pass the 3 cm line in either of the two arms of the olfactometer in 10 min; these were excluded from statistical analysis. The olfactometer was rotated 180° after every three trials, and was washed after every nine trials with water and acetone, and dried at 100°C in an oven. After this procedure, the tissue papers with test substances were renewed. Each treatment was tested at least 40 times.

Sentinel Egg Mass Bioassays These experiments were conducted in two different staked organic tomato fields (*Solanum lycopersicum* L. var. “Big Beef”; flowering and early fruiting stages) at the UCD Student Farm during the summer of 2015. Both fields were surrounded by plantings of sweet alyssum [*Lobularia maritima* L. (Brassicaceae)] as a nectar source for natural enemies.

Euschistus conspersus sentinel egg masses were deployed in 12 randomized complete blocks, each consisting of 4 egg masses per treatment, as follows. Six 90 m row beds spaced on 2 m centers were selected for the study. Four *E. conspersus* sentinel egg masses <24-h old (totaling 50 eggs) from the laboratory colony were fastened onto 1 x 1.5-cm filter paper strips with double-sided tape, then clipped onto nearby plants approximately 1.10 m above the ground at a spacing of 20 m along each bed. Treatments in this experiment included: 1) hexane-extracted eggs with 30 µl of the synthetic aldehyde mixture (0.4 ng/µl, 12 ng total) added, 2) hexane-extracted eggs with 30 µl of methyl (2*E*,4*Z*)-2,4-decadienoate solution (0.1 ng/µl, 3 ng total) added, 3) hexane-extracted eggs and, 4) unextracted eggs. For egg extraction, masses were placed in a glass Petri dish, rinsed with 99% hexane for 5 min, and air dried. Synthetic test compounds were applied to the washed eggs in 30 µl of hexane as indicated above using an electronic pipette (Thermo Fisher Scientific, Walthan, MA, USA). After 24 h the eggs were removed from the field, placed in 7.5 x 1.3 cm glass vials containing a drop of honey and sealed with parafilm, then kept in a laboratory growth chamber under the same conditions as described above. Parasitoid emergence and/or nymphal hatching were checked daily. There were 12 replications for each treatment, totaling 600 exposed eggs per treatment.

Pheromone-baited Traps for Parasitoids The effect of synthetic methyl (2*E*,4*Z*)-2,4-decadienoate on attraction of egg parasitoids was evaluated using yellow sticky traps (13 x 8 cm; Olson Products, Inc., Medina, OH, USA) baited with treated or control rubber septa. Gray rubber septa (9 mm, Soxhlet-extracted overnight with methylene chloride; West Pharmaceutical Services, Kearney, NE, USA) were impregnated with methyl (2*E*,4*Z*)-2,4-decadienoate on the same day they were placed in the field. Initially, stock solutions of methyl (2*E*,4*Z*)-2,4-decadienoate were prepared by serial dilution at concentrations of 10 µg/µl hexane, 1 µg/µl and 0.1 µg/µl; then 100 µl aliquots of the respective stock solutions were applied to prepare sets of septa with 1 mg, 0.1 mg, and 0.01 mg of methyl (2*E*,4*Z*)-2,4-decadienoate per septum; controls consisted of 100 µl of pure hexane/septum. The groups of loaded septa were stored by treatment, wrapped in aluminum foil in plastic freezer bags at -4°C. Septa were transported to the field in Styrofoam boxes with ice packs. The sticky traps were hung from stakes ~1.3 m from the ground every 15 m along four 90 m row beds, each 2 m apart. Fresh sticky traps and lures were replaced daily, with the trap treatment positions randomly assigned. All traps were stored in a refrigerator so that the insects could be counted and identified at a later date. Samples of egg parasitoids were removed from sticky traps using diethyl ether (Sigma-Aldrich) and orange oil (100% essential oil of *Citrus sinensis*, manufactured for Davis Food Co-op, Davis, CA, USA), transferred to vials with ethanol (96%), and sent to Dr. Matthew Buffington (USDA-ARS, Systematic Entomology Laboratory, Washington, D.C., USA) for identification.

Statistical Analyses Olfactometer data were analyzed by χ^2-tests ($P<0.05$), and field data means were compared with one-way ANOVA or by Kruskal-Wallis test, depending on the data normality ($P<0.05$), using BioEstat® 5.0 (Ayres et al. 2007).

RESULTS

Chemical Identifications Methyl (2*E*,4*Z*)-2,4-decadienoate [key ions at *m/z* 67, 79, 81, 111, 151, and 182 (M^+)] was identified as the major volatile component in extracts of *E. conspersus* eggs, as confirmed by retention time and mass spectral matches with the synthetic standard (Fig. 1a). Based on the total ion chromatogram peak area comparisons with the octyl acetate IS, the mean methyl (2*E*,4*Z*)-2,4-decadienoate concentration was 56 ng/g fresh weight of *E. conspersus* eggs (ranging from 20 to 128 ng/g eggs; N=3). In addition, nonanal, (*E*)-2-decenal, (2*E*,4*E*)-2,4-decadienal, (*E*)-2-undecenal, and tridecane were tentatively identified as minor components (Fig. 1a). The common plasticizer, diethylphthalate, was present in all the *E. conspersus* egg extracts as a contaminant; the source of this contaminant is unknown. Palmitic and linoleic acids were abundant non-volatile components in the *E. conspersus* eggs extracts.

The genital capsule extracts of *E. conspersus* males contained two major volatile components, methyl (2*E*,4*Z*)-2,4-decadienoate and tridecane, and a minor component, dodecane (Fig. 2a).

Hexadecanal (63 ng/g), octadecanal (56 ng/g), and eicosanal (5 ng/g) were identified and quantified (via IS; N=2) from *H. halys* egg extracts (Fig. 1B), and their identities were confirmed with the synthetic aldehyde mixture. In addition to relatively large amounts of palmitic and linoleic acids and diethylphthalate, the same minor volatile aldehydes as found in the *E. conspersus* egg extracts (nonanal, (*E*)-2-decenal, (*E*,*E*)-2,4-decadienal and (*E*)-2-undecenal) were also tentatively identified in *H. halys* egg extracts (Fig. 1b).

Analysis of the genital capsule extracts of *H. halys* males revealed the presence of the same three long-chain aldehydes (hexadecanal, octadecanal, and eicosanal) as identified from egg extracts (in a similar ratio) (Fig. 2b). Two other volatile components (tridecane and dodecane) were also detected, plus linoleic acid and some contaminants.

The GC-MS data of both *H. halys* egg extracts and male genital capsule extracts were also examined via extracted ion chromatograms (EIC) for ions characteristic of the two 10,11-epoxy-1-bisabolen-3-ols identified as the male-produced aggregation pheromone of the brown marmorated stink bug [*m/z*: 93, 71, 134, 165, and 220 (M^+)] (Khrimian et al.

2014b). Neither of these pheromone components was detected in either egg or genital capsule extracts of *H. halys*.

Laboratory Bioassays Females of *Te. podisi* and *Tr. erugatus* were both significantly attracted to *E. conspersus* egg extracts versus hexane controls in the olfactometer ($P < 0.05$) (Fig. 3). In contrast, females of *Te. podisi* and *Tr. erugatus* were both significantly repelled by the *H. halys* egg extracts versus hexane controls ($P < 0.05$) (Fig. 4).

When the synthetic compounds were tested in the olfactometer, females of *Te. podisi* and *Tr. erugatus* were both attracted to synthetic methyl (2*E*,4*Z*)-2,4-decadienoate at high (10 ng) and low (1 ng) doses (Figs. 5 and 6). At the high dose of synthetic methyl (2*E*,4*Z*)-2,4-decadienoate, 71% and 23% of *Te. podisi* females responded to the treatment versus the control, respectively ($\chi^2 = 20.00$; df = 1; $P < 0.001$), while 64% and 28% of *Tr. erugatus* females responded to the treatment versus the control, respectively ($\chi^2 = 11.54$; df = 1; $P = 0.0015$) (Fig. 5). At the low dose of synthetic methyl (2*E*,4*Z*)-2,4-decadienoate, 76% and 19% of *Te. podisi* females responded to the treatment versus the control, respectively ($\chi^2 = 28.80$; df = 1; $P < 0.001$), while 56% and 28% of *Tr. erugatus* females responded to the treatment versus the control, respectively ($\chi^2 = 8.00$; df = 1; $P = 0.0095$) (Fig. 6). In contrast, when female wasps were tested in the olfactometer to low doses (0.1 ng) of the synthetic $C_{16,18,20}$ aldehyde blend, females of both species were repelled ($P < 0.05$) (Fig. 7).

Sentinel Egg Mass Bioassays Two scelionid parasitoid species, *Gryon obesum* Masner and *Tr. erugatus* (Hymenoptera: Scelionidae), were obtained from *E. conspersus* sentinel eggs exposed in tomato fields (Table 1). The eggs that were hexane-washed and then treated with methyl (2*E*,4*Z*)-2,4-decadienoate had significantly higher rates of parasitization (94.2%) than the other three treatments ($P < 0.001$). Only 0.2% of washed eggs treated with the $C_{16,18,20}$ aldehyde mixture were parasitized (Table 1, treatment 2), and this level of parasitism was not significantly different from that for the washed eggs plus hexane control (9.7 %; Table 1, treatment 3); parasitism rates for both these treatments were significantly lower than that of unwashed eggs (31.8%, Table 1, treatment 4) (Table 1, P<0.001).

Pheromone-baited Traps for Parasitoids Two parasitoid species, *Tr. basalis* (Hymenoptera: Scelionidae) and *Polynema* sp. (Hymenoptera: Mymaridae), were captured on sticky traps baited with methyl (2*E*,4*Z*)-2,4-decadienoate or with hexane controls. Parasitoid captures decreased daily over the 5-d exposure period of the test. The mean numbers of parasitoids collected in traps baited with 1 mg and 0.1 mg of methyl (2*E*,4*Z*)-2,4-decadienoate were not significantly different from each other (H = 2.5, df = 3, P = 0.50), but were significantly greater than in traps baited with 0.01 mg methyl (2*E*,4*Z*)-2,4-decadienoate or the hexane control (P<0.05). There was no significant difference between the latter two treatments (H = 0.9, df = 3, P = 0.81) (Table 2).

DISCUSSION

The most surprising result of the present study is that the main male-produced aggregation pheromone component of *Euschistus conspersus*, methyl (2E,4Z)-2,4-decadienoate (Aldrich et al. 1991), is the principal volatile on *E. conspersus* eggs, and that females of *Telenomus podisi* and *Trissolcus erugatus* are attracted in olfactometer tests to egg extracts containing low (nanogram levels) of this chemical and to comparable levels of the synthetic compound. Females of *E. conspersus* do not produce methyl (2E,4Z)-2,4-decadienoate (Aldrich et al. 1991). Analyses of extracts of the genital capsules from *E. conspersus* males verified the presence of nanogram levels of methyl (2E,4Z)-2,4-decadienoate. These results suggest that methyl (2E,4Z)-2,4-decadienoate is transferred to conspecific females during mating, and that the surfaces of eggs are contaminated subsequently with this pheromone compound during oviposition, with the parasitoids, thus exploiting this compound as a host egg finding kairomone.

In contrast, eggs of the brown marmorated stink bug, *Halyomorpha halys*, completely lack the sesquiterpenoid pheromone compounds identified from males of this species (Khrimian et al. 2014). However, C16,18,20 aldehydes are present on *H. halys* eggs, and the synthetic mixture of these aldehydes proved to be repellent to *Te. podisi* and *Tr. erugatus* females in olfactometer tests. Furthermore, the same aldehydes were present in extracts of male *H. halys* genital capsules. Comparable analyses were not performed on conspecific females, so the possibility that *H. halys* females themselves are the source of the C16,18,20 aldehydes cannot yet be excluded. More experiments are needed, ideally including *Trissolcus japonicus*, to clarify the semiochemistry of *H. halys* egg parasitism.

Results of field tests using *E. conspersus* sentinel egg masses treated with various extracts or compounds, as well as trials testing traps baited with methyl (2E,4Z)-2,4-decadienoate, substantiated and extended the results of the laboratory olfactometer tests. *Trissolcus erugatus* and another generalist egg parasitoid, *Gryon obesum* Masner (Krupke and Brunner 2003; Rider 2016), were particularly attracted to hexane-washed sentinel eggs of *E. conspersus* that had been treated with methyl (2E,4Z)-2,4-decadienoate. By contrast, hexane-washed egg masses of *E. conspersus* that were treated with the mixture of aldehydes found on *H. halys* eggs were avoided by these parasitoids in sentinel egg field bioassays, suggesting that these compounds serve a defensive role. In addition, sticky traps baited with methyl (2E,4Z)-2,4-decadienoate and deployed in staked tomatoes caught mainly *Trissolcus basalis*, demonstrating that females of this exotic generalist, introduced for biological control of *Nezara viridula* in 1986 (Hoffmann et al. 1991), recognized the key pheromone component of *E. conspersus* (Weber et al. 1996) even though they did not oviposit in, or were unable to survive in *E. conspersus* sentinel eggs.

Both *Telenomus* and *Trissolcus* species are known to exploit the attractant pheromones of their heteropteran hosts as cues (i.e., as kairomones) to guide their long-range search for potential host eggs (Conti and Colazza 2012; Tognon et al. 2014). In particular, *Te. podisi* females were attracted to traps baited with racemic methyl 2,6,10-trimethyltridecanoate (Silva et al. 2006), the main male-produced aggregation pheromone component of *E. heros* (Aldrich et al. 1994; Borges and Aldrich 1994). The study of Borges et al. (1999) is particularly illuminating for *Te. podisi* parasitism of *E. heros* eggs, because the attractiveness of both fertile and unfertilized host eggs to female wasps was studied; *E. heros* females eventually lay eggs if not allowed to mate, which precludes the possibility of male-produced semiochemicals being transferred to these eggs. Borges et al. found that unfertilized *E. heros* eggs were less attractive to, and stimulatory for oviposition by, *Te. podisi* females than were fertilized eggs.

Furthermore, egg-sized glass beads (3 beads/replicate) treated with an extract of fertile eggs, an extract of sexually mature males, or 0.1 ng of synthetic racemic methyl 2,6,10-trimethyltridecanoate were as attractive and stimulatory to the wasps as fertile eggs; higher doses of synthetic pheromone were less attractive (Borges et al. 1999). These findings are consistent with our interpretation that stink bug methyl ester pheromones are produced in the genital capsule of males, transferred to females during mating, and eventually transferred to eggs during oviposition. Nevertheless, Borges et al. (1999) failed to detect methyl 2, 6,10-trimethyltridecanoate in extracts of *E. heros* fertile or infertile eggs, and the same negative result was reported recently by the same group in a study on *T. podisi* parasitism of *E. heros* (Michereff et al. 2016). The discrepancy between the latter researchers' results and our data may be due to the fact that we extracted gram quantities of *E. conspersus* eggs vs. their extraction of only 20 E. heros eggs per sample. Future research is needed to clarify this discrepancy. Be that as it may, it appears that the long-range pheromone from *Euschistus* males may be a relatively easily detectable, but imprecise, cue leading to the location of potential host eggs, whereas the presence of pheromone methyl esters on host eggs themselves may be a faint, but precise indicator of acceptability (Aldrich et al. 1994).

We suggest that our current findings provide an explanation for the earlier results of Tognon et al. (2014) showing that female wasps reared from eggs of the rice stink bug, *T. limbativentris*, prefer to oviposit in eggs of *E. heros* after just one generation on the latter host. We hypothesize that the preference switch to *E. heros* eggs is due to the imprinting of wasps during eclosion to methyl ester pheromone componentsfrom males that are transferred to the surface of *E. heros* eggs via mated females. Inherent in this explanation is that emerging wasps are able to learn to prefer odors on the surface of eggs, which was demonstrated by Tognon et al. (2013) using lemongrass extract applied to the surface of washed host eggs. This hypothesis is reminiscent and pertinent to a modern interpretation (Barron 2001; Corbet 1985) of Hopkins' host selection principle: that many phytophagous and parasitic insects have a preference for the host species on which they developed (Hopkins 1917).

In concluding, it is worthwhile to consider the known distributions of methyl ester vs. sesquiterpenoid pheromones in pentatomids, and the potential ramifications of our current findings for future research on the mediation of egg parasitoid behaviors by heteropteran semiochemicals. Male-produced attractant pheromones have been identified for species in several genera of phytophagous stink bugs, primarily species that are agricultural pests, and many of these pentatomids produce pheromones consisting of methyl esters and/or sesquiterpenoids (Khrimian et al. 2014; Millar 2005; Weber et al. 2014). Studies of egg parasitoids of several species of the "sesquiterpenoid-type" pheromone species have been conducted, especially for the *Nezara viridula*/*Tr. basalis* host/parasitoid pair (Conti and Colazza 2012), yet there is no evidence from any of these species that sesquiterpenoids are transferred to the surface of eggs via mating. In *N. viridula*, males are believed to produce and release their bisabolene epoxide-type sesquiterpenoid pheromone components from single-celled epidermal glands on the abdominal sternum (Cribb et al. 2006), which would explain why their sesquiterpenoid pheromone components are not transferred to conspecific eggs during mating. Our inability to detect the bisabolene-type sesquiterpenes of *H. halys* males in their genital capsules or on the surface of conspecific eggs is consistent with pheromone biosynthesis taking place in the cuticle of the abdominal sternum, as in *Nezara* males. If methyl ester pheromones of other species besides *Euschistus* spp. prove to come from the genital capsules of males, whereas the sesquiterpenoid type pheromones come from the abdominal sternum of males (in species producing those compounds), then our proposed hypothesis for the kairomonal dichotomies discovered for *Telenomus* and *Trissolcus* parasitoids and *Euschistus*, *Tibraca*, and *Halyomorpha* host eggs can be tested further. Moreover, analogous host/egg parasitoid predictions can also be proposed and tested in species in the related families Coreidae and Alydidae, some of which possess socalled ventral abdominal glands positioned such that their secretions are likely to be transferred to females during mating (Aldrich 1988; Millar 2005).

Finally, one may wonder what type of pheromone system evolved first in the Pentatomidae, and why? As noted by Blum (1974), the semiochemical category known as "kairomones", signals defined as maladaptive to the producing individuals, is an evolutionarily artificial category because nothing can evolve if it is solely disadvantageous to the emitter. Therefore, there must be some positive function associated with methyl ester pheromones being transferred to females during mating that, at least in evolutionary history, outweighed the disadvantage of attracting egg parasitoids. One such advantage may be that marking females as mated might limit subsequent matings by conspecific males or the same male himself. However, over time, as parasitoids evolved to exploit this female and egg-marking vulnerability, selection led to the evolution of the alternative sesquiterpenoid pheromone system, possibly through a stage in which species utilized both methyl ester and sesquiterpenoid compounds (e.g., McBrien et al. 2002) in their pheromone blends. In this scenario, the sesquiterpenoid pheromone system would be more derived than the methyl ester pheromone system. Our finding, that instead of transferring sesquiterpene pheromone compounds to females, males of the brown marmorated stink bug produce aldehydes in the genital capsule that may defend against North American egg parasitoids once transferred via mating to conspecific eggs [but not against *Tr. japonicus* with which it coevolved (Herlihy et al. 2016)]. This suggests that evolution toward an "enemy free space" (Jeffries and Lawton 1984) has been a powerful force in heteropteran evolution.

Modern phylogenetics may soon provide an answer as to which pheromone type came first in the Pentatomidae, with research on stink bug phylogenetics in progress (Dr. Jocelia Grazia, Universidade Federal do Rio Grande do Sul, Brazil; pers. Comm.). In the meantime, the current semiochemical knowledge should be useful in guiding biological control of heteropteran pests.

Figure Captions

Fig. 1 Total ion chromatograms for representative analyses of **A)** *Euschistus conspersus* egg extract, and **B)** *Halyomorpha halys* egg extract. Compounds labeled in bold (methyl (2*E*,4*Z*)-2,4-decadienoate for *E. conspersus*; hexadecanal, octadecanal and eicosanal for *H. halys*) were positively identified by comparisons to authentic standards; other compounds were tentatively identified by comparisons to published mass spectra.
* denotes contaminants.

Fig. 2 Total ion chromatograms for representative analyses of **A)** male *Euschistus conspersus* genital capsule extract (n = 5), and **B)** male *Halyomorpha halys* genital capsule extract (n = 5). Compounds labeled in bold (methyl (2*E*,4*Z*)-2,4-decadienoate for *E. conspersus*; hexadecanal, octadecanal and eicosanal for *H. halys*) were positively identified by comparisons to authentic standards; other compounds were tentatively identified by comparisons to published mass spectra. * denotes contaminants.

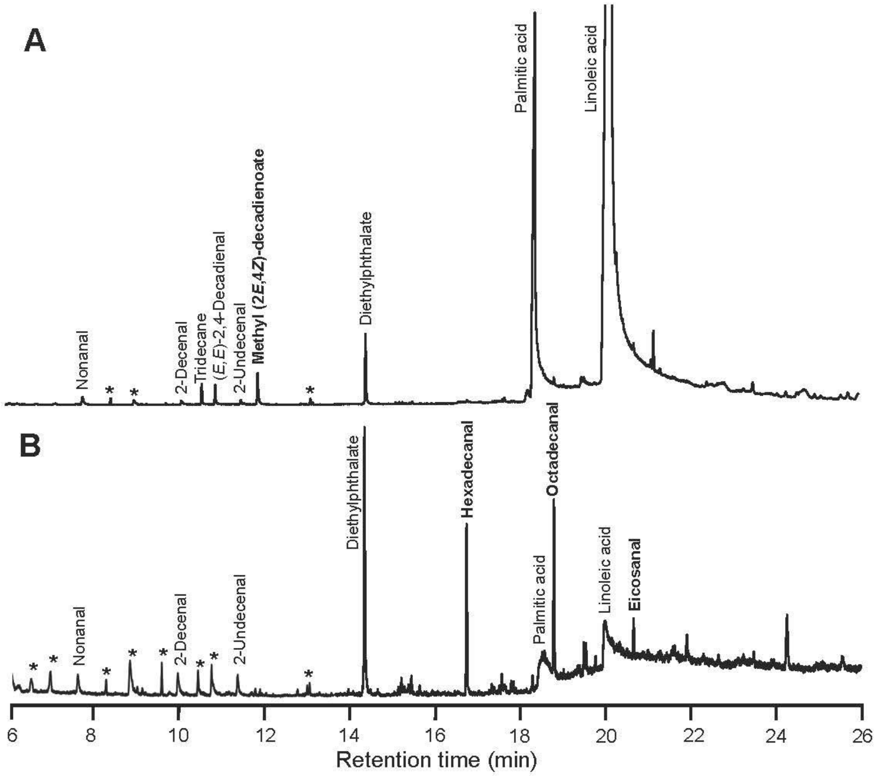
Fig. 3 Olfactometer responses of *Telenomus podisi* and *Trissolcus erugatus* females (reared from *Euschistus conspersus* eggs) to volatiles from *E. conspersus* egg extracts (10 egg equivalents; EE) versus hexane (Numbers of insects are in parentheses; values followed by an asterisk are significantly different by χ^2 at P<0.05 from the hexane control).

Fig. 4 Olfactometer responses of *Telenomus podisi* and *Trissolcus erugatus* females (reared from *Euschistus conspersus* eggs) to volatiles from *Halyomorpha halys* egg extracts (4.5 egg equivalents; EE) versus hexane (numbers of insects are in parentheses; values followed by an asterisk are significantly different by χ^2 at P <0.05 from the hexane control).

Fig. 5 Olfactometer responses of *Telenomus podisi* and *Trissolcus erugatus* females (reared from *Euschistus conspersus* eggs) to methyl (2*E*,4*Z*)-2,4-decadienoate (10 ng) versus hexane (numbers of insects are in parentheses; values followed by an asterisk are significantly different by χ^2 at P <0.05 from the hexane control).

Fig. 6 Olfactometer responses of *Telenomus podisi* and *Trissolcus erugatus* females (reared from *Euschistus conspersus* eggs) to methyl (2*E*,4*Z*)-2,4-decadienoate (1 ng) versus hexane (numbers of insects are in parentheses; values followed by an asterisk are significantly different by χ^2 at $P < 0.05$ from the hexane control).

Fig. 7 Olfactometer responses of *Telenomus podisi* and *Trissolcus erugatus* females (reared from *Euschistus conspersus* eggs) to methyl (2*E*,4*Z*)-2,4-decadienoate (0.1 ng) versus hexane (numbers of insects are in parentheses; values followed by an asterisk are significantly different by χ^2 at $P < 0.05$) from the hexane control).


A
Nonanal
*
*
2-Decenal
Tridecane
(E,E)-2,4-Decadienal
2-Undecenal
Methyl (2E,4Z)-decadienoate
*
Diethylphthalate
Palmitic acid
Linoleic acid
B
*
*
Nonanal
*
*
*
2-Decenal
*
*
2-Undecenal
*
Diethylphthalate
Hexadecanal
Palmitic acid
Octadecanal
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Eicosanal
6
8
10
12
14
16
18
20
22
24
26
Retention time (min)

Figure 1

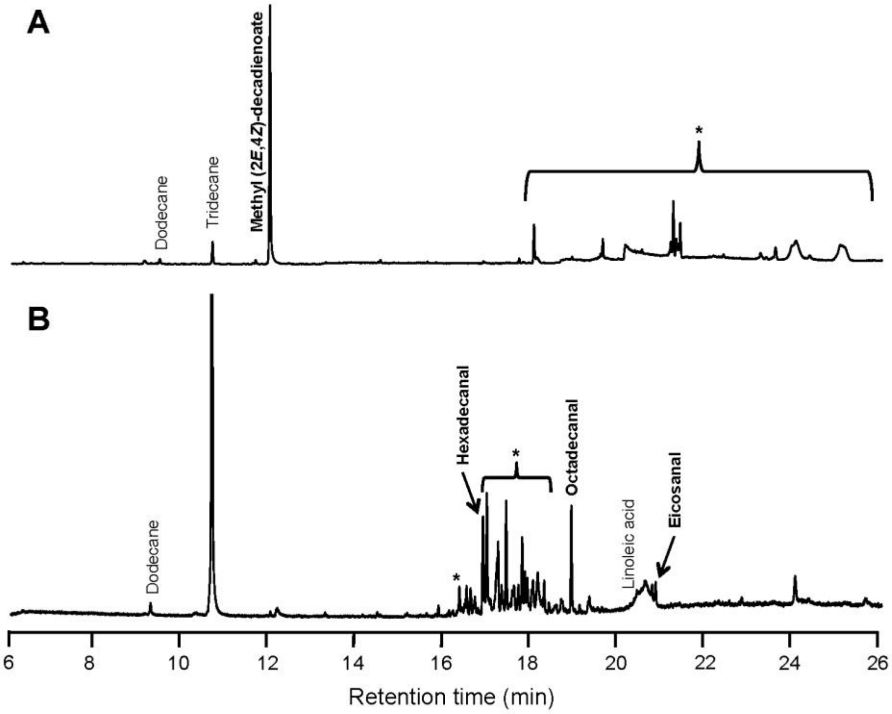
A
Dodecane
Tridecane
Methyl (2E,4Z)-decadienoate
*
B
Dodecane
*
Hexadecanal
*
Octadecanal
Linoleic acid
Eicosanal
6
8
10
12
14
16
18
20
22
24
26
Retention time (min)

Figure 2

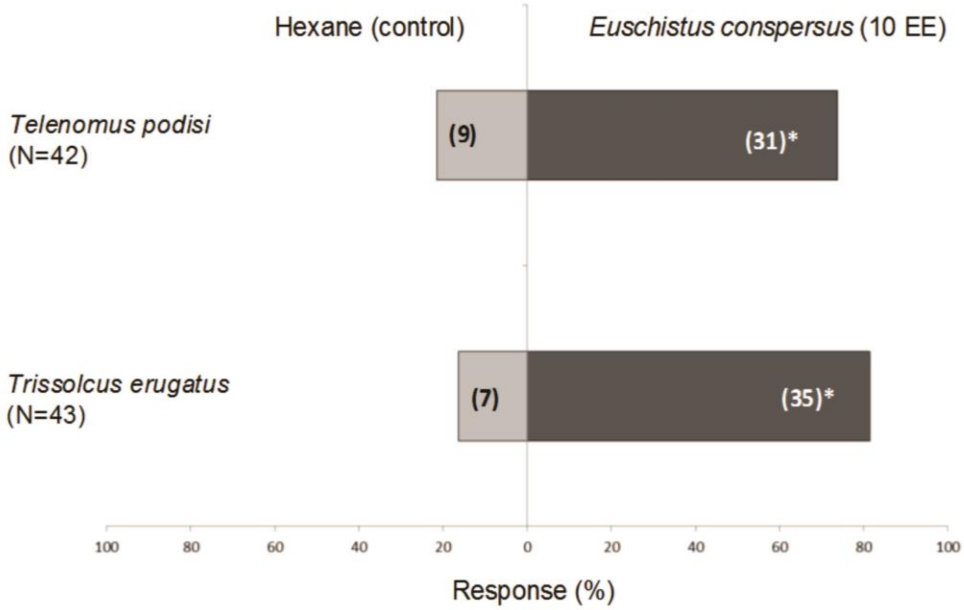
Hexane (control)
Euschistus conspersus (10 EE)
Telenomus podisi
(N=42)
(9)
(31)*
Trissolcus erugatus
(N=43)
(7)
(35)*
100
80
60
40
20
0
20
40
60
80
100
Response (%)

Figure 3.

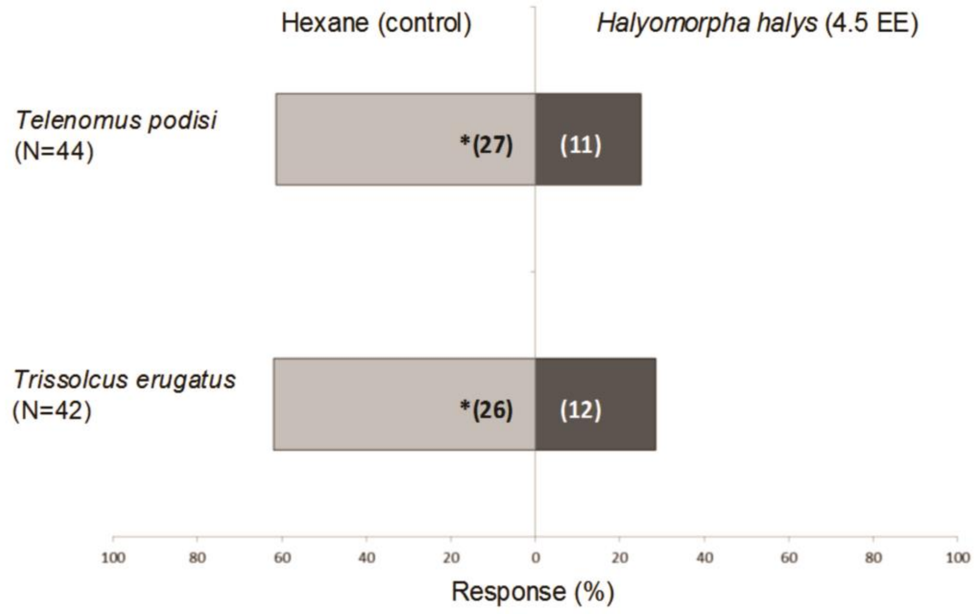
Hexane (control)
Halyomorpha halys (4.5 EE)
Telenomus podisi (N=44)
*(27)
(11)
Trissolcus erugatus (N=42)
*(26)
(12)
100
80
60
40
20
0
20
40
60
80
100
Response (%)

Figure 4

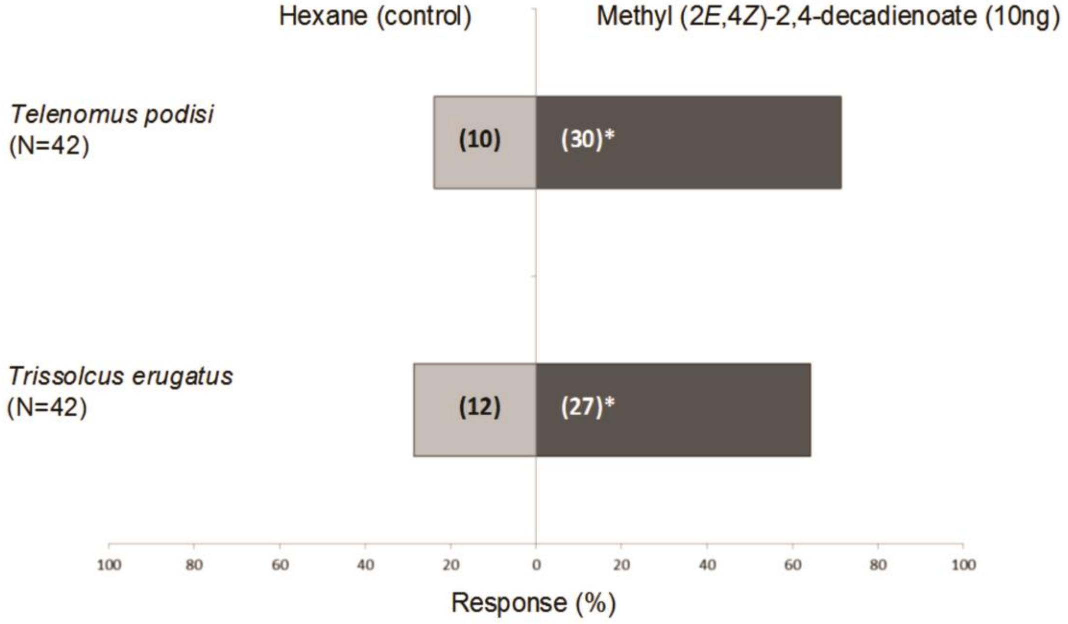
Hexane (control)
Methyl (2*E*,4*Z*)-2,4-decadienoate (10ng)
Telenomus podisi
(N=42)
(10)
(30)*
Trissolcus erugatus
(N=42)
(12)
(27)*
100
80
60
40
20
0
20
40
60
80
100
Response (%)

Figure 5

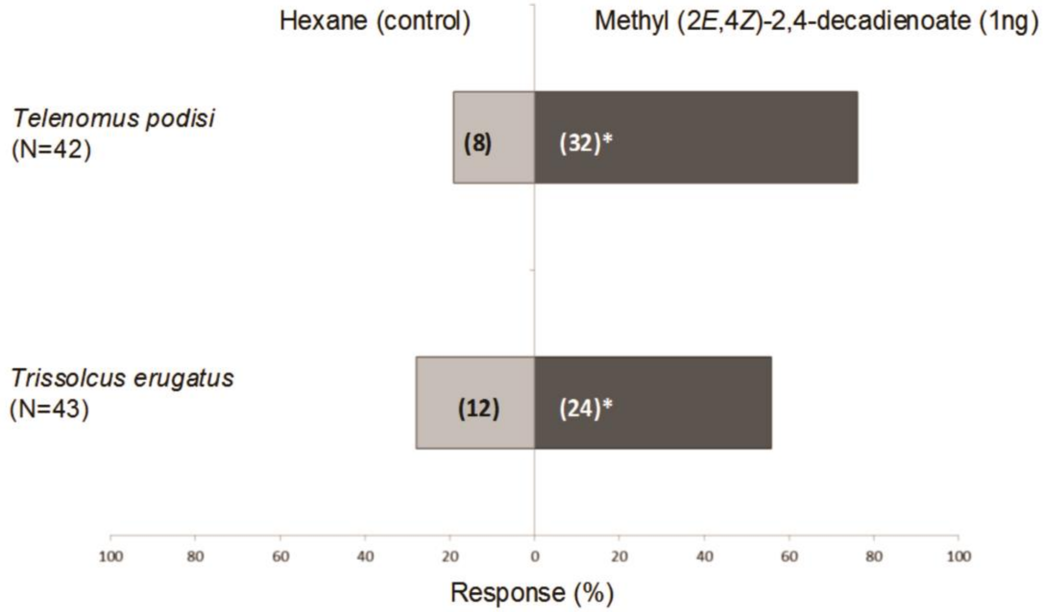
Hexane (control)
Methyl (2E,4Z)-2,4-decadienoate (1ng)
Telenomus podisi
(N=42)
(8)
(32)*
Trissolcus erugatus
(N=43)
(12)
(24)*
100
80
60
40
20
0
20
40
60
80
100
Response (%)

Figure 6

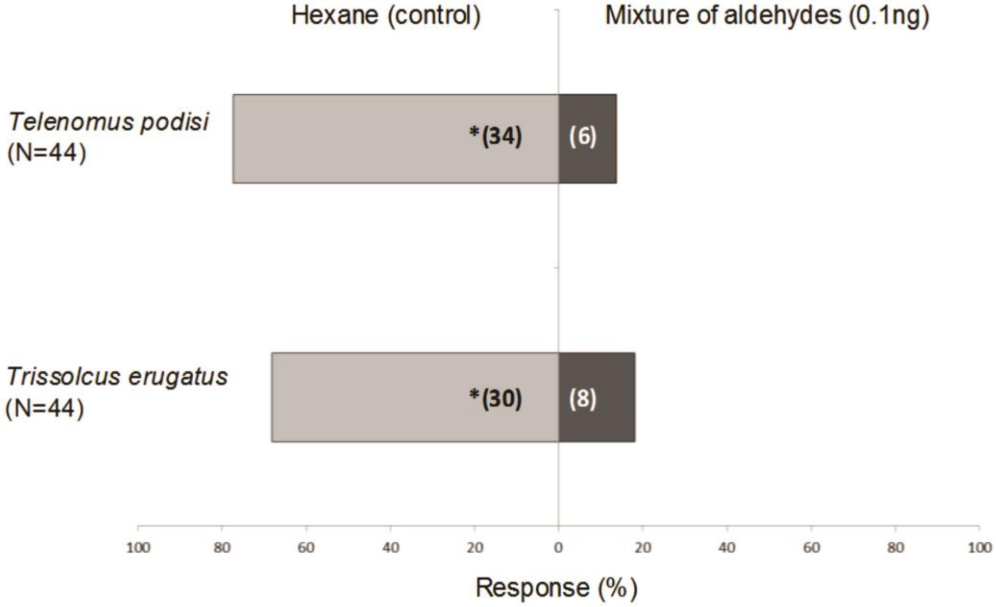
Hexane (control)
Mixture of aldehydes (0.1ng)
Telenomus podisi
(N=44)
*(34)
(6)
Trissolcus erugatus
(N=44)
*(30)
(8)
100
80
60
40
20
0
20
40
60
80
100
Response (%)

Figure 7

Table 1 Field exposure of *Euschistus conspersus* eggs treated as follows: 1) hexane-washed eggs treated with 3 ng methyl (2*E*,4*Z*)-2,4-decadienoate; 2) washed eggs treated with 12 ng $\mathbf{C}_{\mathbf{16,18,20}}$ aldehyde mixture; 3) washed eggs treated with hexane; 4) unwashed eggs

Treatment	No. Eggs exposed	Means of parasitized eggs*	% Parasitized eggs	% Emergence	% Parasitoid species composition	
					Gryon obesum	*Trissolcus erugatus*
1	600	47.08±0.65a	94.2	93.7	53.2	46.6
2	600	0.08±0.08b	0.2	0	—	—
3	600	4.83±1.2b	9.7	9.7	51.9	48.1
4	600	15.91±2.06c	31.8	30.8	44.5	55.5

*Average of parasitized eggs were compared by one-way ANOVA ($P<0.05$)

Table 2 Parasitoids caught in sticky traps baited with the following doses of methyl (2*E*,4*Z*)-2,4-decadienoate or hexane alone: 1) 1 mg/septum; 2) 0.1 mg/septum; 3) 0.01 mg/septum; 4) hexane (control)

Treatments	Days/no. parasitoids collected					Average of parasitoids collected*	No. specimens	
	1st	2nd	3rd	4th	5th		*Trissolcus basalis*	*Polynema* sp.
1	17	8	6	4	2	1.85±0.35a	29	8
2	9	4	5	2	1	1.05±0.18a	16	4
3	2	1	1	0	0	0.2±0.09b	4	0
4	0	1	1	1	0	0.15±0.08b	2	1

*Average of parasitized eggs were compared by Kruskal-Wallis ($P<0.05$)

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6 ARTIGO 4

Halyomorpha halys (Heteroptera: Pentatomidae) egg surface chemicals repel north American *Trissolcus* and *Telenomus* (Hymenoptera: Scelinoidae) parasitoids*

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Roberta Tognon[1*], Jeffrey R. Aldrich[2,4], Matthew L. Buffington[3], Elijah J. Talamas[3], Josué Sant'Ana[1], Frank G. Zalom[4]

[1]Department of Crop Protection, PPG-Fitotecnia, Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 7712, 91540-000 Porto Alegre, RS, Brazil.

[2]Jeffrey R. Aldrich Consulting LLC, 95061, Santa Cruz, CA, USA.

[3]Systematic Entomology Laboratory, Agricultural Research Service, U.S. Department of Agriculture, c/o National Museum of Natural History, Smithsonian Institution, P.O. Box 37012, MRC-168, Washington, DC 20013-7012 USA.

[4]Department of Entomology and Nematology, University of California, Davis, One Shields Avenue, 95616, Davis, California, USA.

*Corresponding author:
Email address: roberta.tognon@ufrgs.br
Postal address: Universidade Federal do Rio Grande do Sul (UFRGS), Faculdade de Agronomia, Departamento de Fitossanidade, Av. Bento Gonçalves, 7712, 91540-000 Porto Alegre, RS, Brazil

Abstract

Halyomorpha halys (Stål) (Heteroptera: Pentatomidae), the brown marmorated stink bug (BMSB), is an invasive pest in the U. S., where it has been largely unaffected by natural enemies. Using rinsed and unrinsed frozen and unfrozen egg masses in laboratory bioassays, we studied how surface chemicals on the eggs of *H. halys* might suppress parasitism by the native North American scelionid parasitoids, *Telenomus podisi* Ashmead and *Trissolcus erugatus* Johnson (Hymenoptera: Scelionidae). Parasitism of unfrozen hexane-rinsed and unrinsed *H. halys* eggs was not observed in laboratory bioassays. However, both *Te. podisi* and *Tr. erugatus* parasitized significantly more frozen rinsed eggs than frozen unrinsed eggs. Some of the parasitoids of both species that successfully emerged from frozen rinsed and unrinsed eggs were subsequently able to develop in unfrozen *H. halys* eggs for at least four generations. We conclude that the success of indigenous egg parasitoids in successfully parasitizing the invasive *H. halys* in North America may be limited by compounds present on the surface of BMSB eggs, and that native parasitoids can learn to recognize the exotic BMSB eggs under specific circumstances. Knowledge of the semiochemistry of egg parasitism increases the likelihood that it may be possible to artificially select native parasitoids for biological control of *H. halys* and other invasive pests.

Keywords: Brown marmorated stink bug, *Trissolcus erugatus*, *Telenomus podisi*, Telenominae, Semiochemistry, Kairomone

1. Introduction

Halyomorpha halys, the brown marmorated stink bug (BMSB), is native to eastern Asia, and over the last two decades this species has become an invasive pest in North America and Europe (Hoebeke and Carter, 2003; Fogain and Graff, 2011; Rice et al., 2014; Gariepy et al., 2015). In the U. S. *H. halys* was first recorded in 1996, in Allentown, Pennsylvania (Hoebeke and Carter, 2003). Since its establishment and spread in the mid-Atlantic states, and subsequently elsewhere in North America and Europe, this highly polyphagous pest

(Lee et al., 2013) has caused significant economic losses to many crops including peaches, apricot, plum, apples, pears, persimmons, grapes, green beans, peppers, eggplant, tomatoes and sweet corn to name a few (Rice et al., 2014; Kuhar et al., 2012; Leskey et al., 2012b; Cissel et al., 2015; Joseph et al., 2015; Bariselli et al., 2016).

While insecticides are currently the most widely used control tactic for *H. halys* in its introduced range, the most effective insecticides also adversely affect natural enemy populations (Leskey et al., 2012a). Developing strategies for conserving or augmenting *H. halys* parasitoids will be necessary to achieve sustainable control (Côrrea-Ferreira and Moscardi, 1995; Consoli et al., 2010; Sithanantham et al., 2013). In Asia, hymenopteran parasitoids in the genera *Trissolcus* and *Telenomus* (both Scelionidae), *Ooencyrtus* (Encyrtidae) and *Anastatus* (Eupelmidae) parasitize eggs of *H. halys*, with *Trissolcus japonicus* (Ashmead) and *Trissolcus cultratus* Mayr. (Chinese strain) being the most important parasitoids in the native BMSB range (Yang et al., 2009; Talamas et al., 2015a). However, in Europe (Haye et al., 2015) and North America (Rice et al., 2014; Talamas et al., 2015a; Herlihy et al., 2016; Lara et al., 2016) species in the aforementioned genera develop poorly or not at all on *H. halys* eggs, contributing to the population outbreaks reported from these regions (Abram et al., 2014). Haye et al. (2015) studied the effects of freezing *H. halys* egg masses on parasitism by native egg parasitoids in Europe; freezing eggs for a few minutes at -80ºC enabled the scelionids *Trissolcus semistriatus* (Nees) and *Tr. cultratus* (Swiss strain), and the eupelmid *Anastatus bifasciatus* (Geoffroy), to complete their development.

Successful parasitism involves both chemical and physical cues (Conti and Colazza, 2012), but in nature parasitoids are presented with a great complexity of odors. To overcome this, parasitoids have evolved semiochemical search strategies to increase their host-finding efficiency (Vinson, 1985; Vet and Dicke, 1992; Vet et al., 1995; Vinson, 1998; Steidle and van Loon, 2002; Fatouros et al., 2008;). For examples, attractant and contact pheromones of host adults are exploited by some egg parasitoids as long- and short-range kairomones, respectively, to home-in on areas likely to have host eggs (Borges et al., 1998; Bruni et al., 2000; Conti and Colazza, 2012; Hilker et al., 2000; Aldrich et al., 2007; Tognon et al., 2014). Some parasitoid species recognize the adhesive material that holds pentatomid egg masses together and to the substrate as a host-finding kairomone (Papaj and

Lewis, 1993; Quicke, 1997; Sasaki and Godfray, 1999; Steidle and van Loon, 2002). In addition, heteropteran egg parasitoids also use chemical compounds present on the surface of host eggs as reliable host location and recognition kairomones (Renou et al., 1992; Vet and Dicke, 1992; Bin et al., 1993; Tognon et al., 2014).

In the present study, we hypothesize that chemicals present on eggs of the exotic BMSB may prevent parasitism by endemic parasitoids found in its introduced range. We further propose that endemic parasitoids can learn to recognize BMSB eggs under specific circumstances. We tested these hypotheses by evaluating parasitism success of the endemic North American parasitoids, *Te. podisi* and *Tr. erugatus*, to *H. halys* egg masses that were alive or frozen, and either rinsed with solvent to remove surface chemicals or not rinsed. Subsequently, female parasitoid wasps that emerged from frozen-rinsed and frozen-unrinsed *H. halys* egg masses were used for additional no choice parasitism selection experiments. Better understanding of the chemical ecology of exotic stink bug eggs and their role in preventing successful parasitism by endemic parasitoids may lead to the artificial selection of strains capable of developing in fresh eggs of *H. halys* and other invasive pests.

2. Materials and methods

2.1. Origin and maintenance of stink bug and parasitoid colonies

Halyomorpha halys nymphs and adults were collected from Fremont Community Garden in Sacramento, CA USA during summer 2015. The adults were reared in plastic cages (19x25x19cm) in a Percival Model I-36LL growth chamber (Perry, IA, USA) at 26±1°C, 65±10% RH, 16L:8D photoperiod in the Zalom laboratory at the University of California, Davis (UC Davis). They were supplied with water in a glass shell vial with a cotton wick, and fed with organic green beans, tomatoes, sunflower and pumpkin seeds, and sliced apple, plum or cherry. Paper towels served as an oviposition substrate. Eggs were collected daily for use in bioassays or kept under the same adult colony conditions in separate nymphal rearing cages. Newly eclosed adults were removed daily from the nymphal cages and transferred to separate cages containing recently emerged adults. Each cage contained no more than 50 adults and the food was replaced twice weekly.

Nymphs and adults of *Euschistus conspersus* Uhler (Heteroptera: Pentatomidae) were obtained from Paramount Farming in Shafter, Kern Co., CA, USA, in December 2014 from collections made in that area during summer 2014, and were maintained as describe above for *H. halys*. In addition to collecting their eggs for colony maintenance and bioassays, some of the eggs were also used as hosts for parasitoid colonies.

A colony of the spined soldier bug, *Podisus maculiventris* Say (Heteroptera: Pentatomidae), was established from adults collected at the arboretum of the University of California at Santa Cruz (UCSC) in Rescue® Stink Bug Traps (Sterling International, Inc., Spokane, WA, USA) baited with the synthetic aggregation pheromone of *P. maculiventris* (Aldrich et al., 1984). The *P. maculiventris* colony was maintained as described for the other stink bugs in the Zalom laboratory at UC Davis except that they were fed larvae and pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) obtained commercially (Rainbow Mealworms, Compton, CA, USA). Eggs were collected daily and allowed to hatch for colony maintenance, or used as hosts for maintaining the *Te. podisi* colony.

Our *Tr. erugatus* were originally collected at the Student Farm at UCD using fresh *P. maculiventris* egg masses clipped onto the leaves of garden vegetables, and *Te. podisi* were collected at the UCSC arboretum using fresh *P. maculiventris* egg masses clipped to leaves of coast live oaks (*Quercus agrifolia*). After 48 h the sentinel eggs were removed from the field, and returned to the laboratory where they were held in 7.5x1.3 cm glass tubes at room temperature containing a drop of honey until adult parasitoids emerged. *Trissolcus erugatus* was identified using the key of Talamas et al. (2015b) and *Te. podisi* using the key of Johnson (1984). Both *Tr. erugatus* and *Te. podisi* were maintained in a Percival Model I-36LL growth chamber (Perry, IA, USA) in the Zalom laboratory at UC Davis at 26±1°C, 65±10% RH, and 14L:10D photoperiod using *E. conspersus* eggs as host, and provided with a drop of honey every 3 days.

Specimens of both egg parasitoids were sent to the Systematic Entomology Laboratory, USDA-ARS, Washington D.C., for identification; voucher specimens are deposited in the National Insect Collection, National Museum of Natural History, Smithsonian Institution.

Voucher specimens for both the parasitoids and the pentatomid species have been deposited in the R. M. Bohart Museum of Entomology at UC Davis.

2.2. No-choice laboratory parasitism tests

We compared the ability of *Te. podisi* and *Tr. erugatus* to parasitize *H. halys* eggs using a no choice bioassay. The bioassays were performed in growth chambers under the same conditions used for rearing the stink bugs. The treatments included: 1) live eggs; 2) frozen eggs; 3) live eggs rinsed with hexane and; 4) frozen eggs rinsed with hexane.

Egg masses of *H. halys* were collected each day from the laboratory colony, and all eggs used for the bioassay were less than 12 h old. For the bioassays, an egg mass with 10 eggs was glued onto a 1x1.5 cm filter paper strip using double-sided sticky tape, and placed in a 7.5x1.3 cm glass tube containing a drop of honey. A 24-h-old female that was paired with males since emergence (i.e. mated, according Yeargan, 1982) and without previous oviposition experience with either *Te. podisi* or *Tr. erugatus*, was introduced into a tube with the test eggs. The tube containing the wasp and test eggs was then sealed with Parafilm® (Bemis Flexible Packaging, Neenah, WI) for 24 hours, after which the exposed eggs were retrieved. These egg masses served as live, unrinsed controls for the bioassays. The same procedure was used to provide egg masses for the other treatments prepared prior to exposure to the female parasitoids.

For the frozen-egg treatments, fresh *H. halys* egg masses were collected from the colony and placed into an ultra cold freezer (Thermo Scientific Forma 900 Series, Pittsburgh, PA, USA) at -80ºC for 15 min on the day of collection before exposing them to the parasitoids. For hexane-rinsed egg treatments, fresh egg masses were placed in a glass Petri dish and submerged in 99% hexane (Sigma-Aldrich, St. Louis, MO, USA) for 5 min. The eggs were then air dried and used for the parasitoid bioassays. Eggs used for the rinsed, frozen-egg treatment were subjected to the additional step of freezing as described previously before exposure to female parasitoids.

Female wasps that emerged from frozen-rinsed and frozen-unrinsed *H. halys* egg masses were used for an additional no choice parasitism selection experiments as follows. Fresh egg masses were removed from the *H. halys* colony and divided into two unfrozen (i.e. alive) groups; one group was rinsed with hexane as previously described and the second one remained unrinsed. Each egg mass (n=10 eggs per mass) was placed into a 7.5 x 1.3 cm glass tube containing a drop of honey and a 24-h-old mated female. Both *Te. podisi* or *Tr. erugatus* that had emerged from the first generation experimental treatments were introduced for 24 h as for the previous generation. The no choice selection was repeated with parasitoids that emerged for additional generations. *Halyomorpha halys* egg masses that were not exposed to any parasitoids were placed separately into 7.5 x 1.3 cm glass tubes as controls. All unhatched eggs were dissected and the presence of parasitoid embryos, if any, was registered as parasitized eggs. Egg mortality was observed when neither parasitoids nor stink bug embryos were found after dissection. There were 10 eggs per mass, and at minimum 20 replicate egg masses were used for each treatment.

3. Statistical analyses

Data were first analyzed for normality, and found not to be normally distributed. Mean egg parasitism, parasitoid emergence, stink bug nymphs emerging, and egg mortality were compared within treatments with a Kruskal-Wallis H-test that indicated significant treatment differences, then subsequently compared by Dunn's test at $P < 0.05$ with BioEstat® 5.0 (Ayres et al. 2007).

4. Results

4.1. No-choice laboratory parasitism tests

Neither *Te. podisi* nor *Tr. erugatus* parasitized unfrozen *H. halys* eggs, whether or not the eggs were rinsed with hexane. However, both species successfully parasitized recently laid *H. halys* eggs that were frozen. *Telenomus podisi* females parasitized a significantly greater percentage of rinsed and frozen eggs (59.6%) than frozen eggs that were not rinsed (27.9%) (H = 19.51, df = 1, $P < 0.0001$). Parasitoid emergence was greater from the rinsed, frozen eggs (46.5%) than from the frozen eggs that were not rinsed (17.9%) (H = 22.29, df = 1, $P < 0.0001$). The percentage of BMSB nymphs emerging was statistically different for the fresh eggs that were not hexane-rinsed (93.7%) than comparable rinsed eggs (50.3%) (H = 35.15, df = 1, $P < 0.0001$); however, overall egg mortality was greater for the fresh, rinsed eggs (49.7%) than for the fresh eggs that were not rinsed (6.7%) (H = 33.76, df = 1, $P < 0.0001$) (Table 1). Results of the no-choice bioassay with *Tr. erugatus* females were similar to that for the *Te. podisi* females. Percent parasitism of frozen *H. halys* eggs was statistically greater for rinsed eggs (80.0%) than for eggs that were not rinsed (49.7%) (H = 19.9, df = 1, $P < 0.0001$). *Trissolcus erugatus* emergence was 71.1% from rinsed, frozen eggs and 46.5% from frozen eggs that were not rinsed (H = 13.96, df = 1, $P = 0.0002$). *Halyomorpha halys* egg mortality was significantly greater for the fresh eggs that were rinsed than for the fresh, unrinsed eggs ($P < 0.0001$) (Table 1).

The hexane solvent had no impact on the emergence of *H. halys* nymphs. There was no significant difference ($P > 0.05$) between the percent nymphal emergence from fresh unexposed eggs that were not rinsed (97.9%) and those that were rinsed (97.2%), nor the percent egg mortality for unexposed not rinsed (2.1%) and those unexposed rinsed (2.8%). No nymphs emerged from any of the frozen eggs (Table 1).

Fresh rinsed and unrinsed BMSB eggs were offered to the female parasitoids that emerged from the *H. halys* eggs exposed during the first experiment (Table 2). In this case, *Te. podisi* parasitized more rinsed eggs (28.3%) than unrinsed eggs (7.9%) (H = 11.71, df = 1, $P = 0.0006$). *Telenomus podisi* emergence was low, and there was no difference between percent emergence from rinsed eggs or unrinsed eggs ($P > 0.05$). However, percent *H. halys* nymphal emergence from unrinsed eggs was greater than from rinsed eggs ($P < 0.05$). By contrast, percent egg mortality was lower for the unrinsed eggs (23.8%) than for the rinsed eggs (45.6%) (H = 11.57, df = 1, $P = 0.0007$) (Table 2). Results were similar for *Tr. erugatus*. There was greater parasitism of rinsed eggs (33.3%) than unrinsed eggs (7.6%) (H = 7.66, df = 1, $P < 0.0056$), and successful parasitoid emergence closely followed the rate of parasitism. Consequently, *H. halys* nymphal emergence from unrinsed eggs (57.2%) was greater than that from rinsed eggs (12.3%) (H = 26.09, df = 1, $P < 0.0001$). Egg mortality was lower for the unrinsed eggs than the rinsed eggs ($P < 0.05$) (Table 2). Similar to the first bioassay, there was no difference in *H. halys* nymphal emergence between fresh unrinsed eggs and fresh rinsed eggs ($P > 0.05$) for control egg masses that were not exposed to parasitoids (Table 2).

Fresh, unrinsed BMSB eggs were offered to the female parasitoids emerging from *H. halys* eggs thereafter for up to four additional generations, yielding the third, fourth, fifth, and sixth (*Tr. erugatus* only) generations of endemic parasitoids that had originally emerged from *H. halys* eggs that were initially exposed to parasitoids during the first experiment. Percent egg parasitism by *Te. podisi* females increased again in the third generation, but decreased in the subsequent two generations. Similar results were observed for percent parasitoid emergence, and there was no emergence at all in the fifth generation. Percentage of eggs with *H. halys* nymphs emerging and percent egg mortality were not significantly different ($P > 0.05$) among these generations (Figure 1).

As had been observed for *Te. podisi*, percent parasitism of fresh *H. halys* eggs by *Tr. erugatus* females and percent parasitoid emergence increased in the third generation, and then significantly declined in the fourth generation. However, the amount of parasitism increased again for the fifth generation before falling again for the sixth. Also, similar to *Te. podisi*, the percentage of eggs with *H. halys* nymphs emerging and percent egg mortality were not significantly different ($P > 0.05$) among these generations (Figure 2).

5. Discussion

Our bioassay results demonstrate that briefly washing *H. halys* eggs with hexane and killing them by freezing enables the native *Telenomus* and *Trissolcus* parasitoids utilized in this study to successfully recognize and parasitize the eggs, and renders them biochemically suitable for development of these parasitoids. It is generally known that scelionid egg parasitoids of Heteroptera (and other insects) must find and parasitize host eggs within two days of oviposition in order to avoid the biochemical developmental barriers presented by the embryos (Vinson, 1998; Conti and Colazza, 2012). Apparently, killing eggs by freezing eliminates the embryonic defenses of *H. halys* eggs while retaining their nutritional competence for complete parasitoid embryogenesis, as has been shown by others (e.g. Haye et al., 2015; Talamas et al., 2015a). Indeed, freezing and storage of the eggs of another stink bug, *Nezara viridula* (Linnaeus) (Heteroptera: Pentatomidae), (Corrêa-Ferreira and Moscardi, 1993) was instrumental in the highly successful program to control this soybean pest in Brazil by augmentation of the scelionid *Tr. basalis* (Corrêa-Ferreira and Moscardi, 1996). However, the finding that volatile compounds on the surface of *H. halys* eggs reduces parasitism by non-adapted egg parasitoids is novel. Certain heteropteran eggs are known to contain repellant compounds (Hinton, 1981; Gillot, 2002).

Recently we investigated the semiochemistry of egg volatiles with the same egg parasitoids as in the present study with *H. halys* eggs and also including eggs of the native stink bug, *E. conspersus* (Tognon et al., 2016). The results of this companion study provide a chemical basis for the divergent responses of these parasitoids to egg volatiles of the native host versus those from the exotic *H. halys* eggs. Females of both parasitoids were highly attracted at short-range to nanogram levels of *E. conspersus* egg volatiles but, as reported herein, females of both wasps were not attracted by volatiles from *H. halys* eggs. Surprisingly, the principal egg volatile found on *E. conspersus* eggs was the main attractant pheromone component produced by *E. conspersus* males, methyl (2*E*,4*Z*)-2,4-decadienoate. Since *Euschistus* male-specific methyl ester pheromones are produced on the inner surface of the genital capsule (Aldrich, unpublished data and Tognon et al. 2016), evidently residues of the male-specific methyl ester pheromone components are transferred onto the eggs via mated females and exploited by parasitoids as a host-finding recognition kairomone (Tognon et al., 2016). On the other hand, the male-produced sesquiterpenoid attractant pheromones of Heteroptera (Millar, 2005), including that for *H. halys* (Khrimian et al., 2014), are apparently released from cells in the abdominal epidermis (Cribb et al., 2006), which explains the absence of these pheromone compounds on the eggs of *H. halys* (Tognon et al., 2016). Although there was no trace of the sesquiterpenoid pheromone components on the eggs of *H. halys*, a mixture of $C_{16,18,20}$ aldehydes (hexadecanal, octadecanal and eicosanal) was identified from rinses of BMSB eggs (Tognon et al., 2016). Aldehydes are reactive compounds that are key components of the defensive secretions (allomones) of nymphs and adults for most of the so-called “true bugs” (Heteroptera) (Aldrich, 1988; Millar, 2005). Many true bugs, particularly stink bugs (Pentatomidae), emit secretions from metathoracic glands in adults and dorsal abdominal glands in nymphs containing $C_{6,8,\text{ or }10}$-α-β-unsaturated straight-chain aldehydes (Aldrich, 1988; Millar, 2005). *(E)*-2-Decenal was one of the first compounds identified from the scent glands of a bug, *N. viridula* (Gilby and Waterhouse, 1965), and was also one of the first compounds identified as a kairomone for a heteropteran egg parasitoid (Mattiacci et al., 1993). Mattiacci et al.

(1993) showed that *Tr. basalis* females were attracted to *(E)*-2-decenal, and that this compound stimulated the oviposition behavior of the wasps, even though no 2-decenal isomers were detected in extracts from *N. viridula* eggs. In fact, *(E)*-2-decenal is the major alk-2-enal in the scent gland secretion of *H. halys* adults (Baldwin et al., 2014), and decenal was a minor aldehyde relative to the $C_{16,18,20}$ aldehydes identified from rinses of *H. halys* eggs (Tognon et al., 2016).

The most important egg parasitoids of *H. halys* in Asia are *Tr. japonicus* and *Tr. cultratus* (Mayr) (Rice et al., 2014; Lara et al., 2016), but the success of endemic parasitoids against this pest in regions it has recently invaded has been limited (Haye et al., 2015; Talamas et al., 2015a; Herlihy et al., 2016). Haye et al. (2015) found that the eupelmid *Anastatus bifasciatus* (Geoffroy) successfully parasitized *H. halys* eggs, and they considered *A. bifasciatus* to be the only potential candidate for augmentative biological control in Europe at this time. In North America, an established population of *Tr. japonicus* successfully parasitizing *H. halys* was first reported in Maryland by Talamas et al. (2015a). More recently, four native hymenopteran species, including three scelionids, *Te. podisi*, *Tr. euschisti* (Ashmead) and *Tr. brochymenae* Ashmead, and the eupelmid *Anastatus reduviid* (Howard), successfully emerged from sentinel *H. halys* eggs in Maryland (Herlihy et al., 2016). In the latter study, emergence of native parasitoids was far greater from frozen eggs than freshly laid live eggs.

Based on the present results, we conclude that the success of indigenous egg parasitoids against the invasive *H. halys* in North America and Europe may be limited by defensive compounds present on BMSB eggs. We observed that some scelionid species endemic to the invasive range of *H. halys* can somewhat overcome the embryonic biochemical egg defenses of this new potential host. In our study, two endemic New World parasitoids that successfully emerged from frozen *H. halys* egg masses from which egg-surface defensive chemicals were removed by solvent rinsing subsequently successfully completed their development in young, live *H. halys* eggs for four (*Te. podisi*) and six (*Tr. erugatus*) generations.

Finally, some authors consider that exotic species act as a trap for endemic parasitoids because they expend energy and resources that will not result in offspring production (Keeler and Chew, 2008; Abram et al., 2014; Haye et al., 2015). On the other hand, although invasive pests suffer low parasitism by endemic species initially, over time they are usually successfully attacked by a greater number of native parasitoids (e.g. Cornell and Hawkins, 1993). We believe that the manipulations reported herein resulting in both *Te. podisi* and *Tr. erugatus* wasps partially overcoming *H. halys* egg defenses is a demonstration of how semiochemical research on egg parasitism may eventually lead to artificial selection of native parasitoids for biological control of invasive pests.

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Author contribution statement

RT, JRA, FGZ and JS conceived and designed the research, RT conducted the experiments and analyzed data. JRA and FGZ contributed to various aspects of the experiments, data interpretation, and editing several drafts of the manuscript. MLB and EJT identified the parasitoids. All authors contributed to writing the paper.

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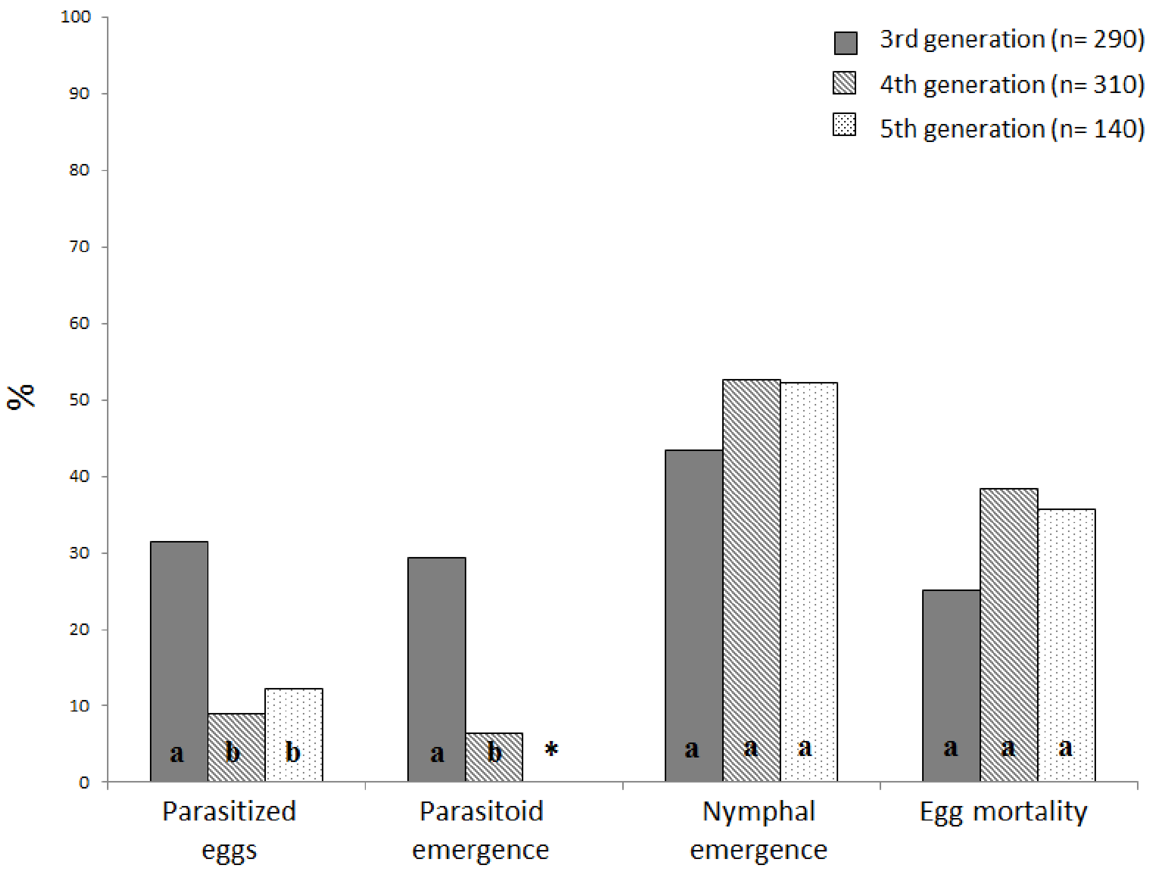
Figure Captions

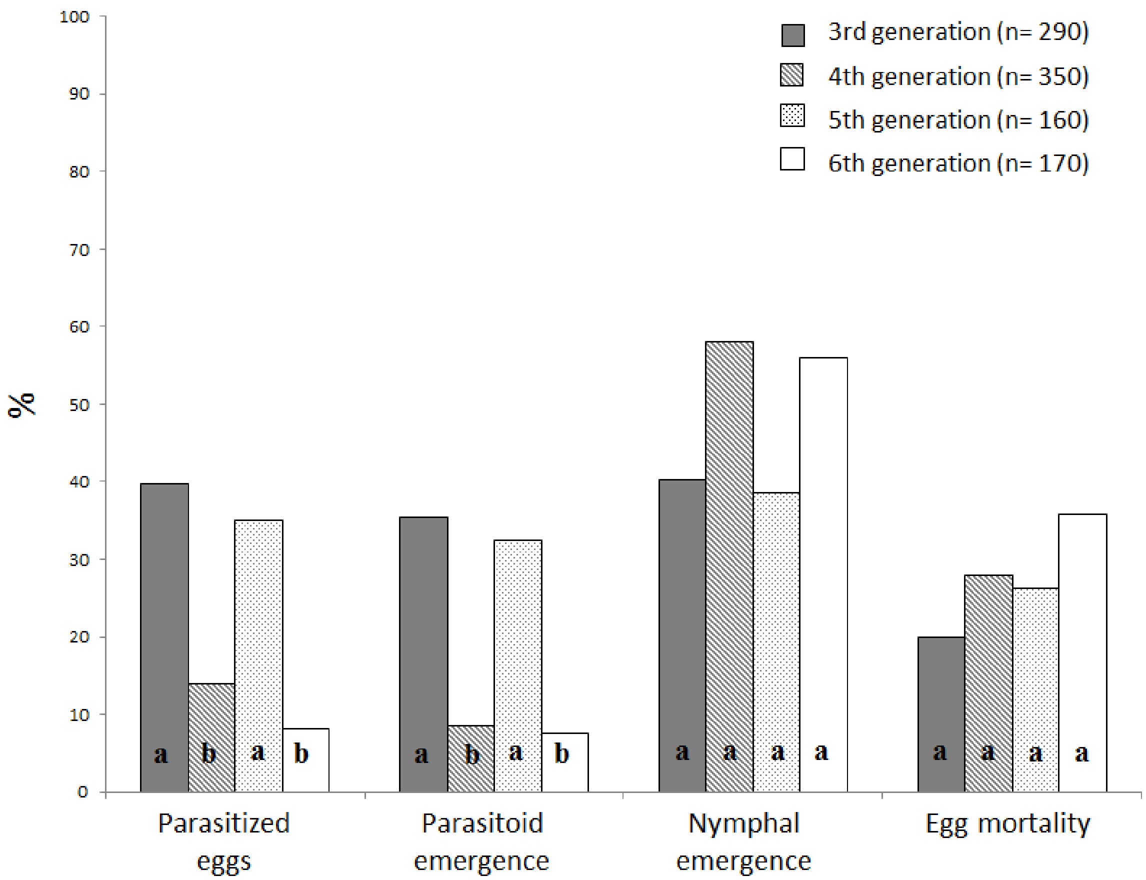
Fig. 1 Percentage of *Halyomorpha halys* eclosion, egg mortality and *Telenomus podisi* parasitism/emergence from fresh eggs exposed to parasitism for five consecutive generations. Bars followed by different letters among generations, in each parameter, are significantly different (Kruskal-Wallis followed by Dunn's test, $P < 0.05$). *No parasitoid emergence

Fig. 2 Percentage of *Halyomorpha halys* eclosion, egg mortality and *Trissolcus erugatus* parasitism/emergence from fresh eggs exposed to parasitism for six consecutive generations. Bars followed by different letters among generations, in each parameter, are significantly different (Kruskal-Wallis followed by Dunn's test, $P < 0.05$)



100
90
80
70
60
50
40
30
20
10
0
%
3rd generation (n= 290)
4th generation (n= 310)
5th generation (n= 140)
a
b
b
a
b
*
a
a
a
a
a
a
Parasitized eggs
Parasitoid emergence
Nymphal emergence
Egg mortality

Fig. 1


100
90
80
70
60
50
40
30
20
10
0
%
3rd generation (n= 290)
4th generation (n= 350)
5th generation (n= 160)
6th generation (n= 170)
a
b
a
b
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b
a
b
a
a
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a
a
a
a
a
Parasitized eggs
Parasitoid emergence
Nymphal emergence
Egg mortality

Fig. 2

Table 1 Parasitism by *Telenomus podisi* and *Trissolcus erugutus* resulting from laboratory no-choice bioassays of *Halyomorpha halys* eggs that were exposed to four treatments *

	No. exposed eggs	Treatments	Mean parasitized eggs**	Mean parasitoid emergence**	Mean BMSB nymphal emergence**	Mean egg mortality** [a]
Telenomus podisi	300	Unrinsed, not frozen	0	0	9.33±0.26a	0.66±0.26a
	300	Rinsed, not frozen	0	0	5.16±0.34b	4.96±0.35b
	260	Rinsed and frozen	5.96±0.5a	4.65±0.45a	0	-
	290	Unrinsed and frozen	2.79±0.32b	1.79±0.18b	0	-
Trissolcus erugatus	310	Unrinsed, not frozen	0	0	9.29±0.24a	0.7±0.24a
	310	Rinsed, not frozen	0	0	6.96±0.26b	3.03±2.9b
	280	Rinsed and frozen	8±0.45a	7.1±0.46a	0	-
	290	Unrinsed and frozen	4.93±0.43b	4.65±0.42b	0	-
Unexposed eggs	190	Unrinsed, not frozen	-	-	9.78±0.02a	0.21±0.02a
	180	Rinsed, not frozen	-	-	9.72±0.03a	0.27±0.03a
	197	Rinsed and frozen	-	-	0	-
	200	Unrinsed and frozen	-	-	0	-

* Means (±SE) for each species/treatment pair within columns for *H. halys* eggs that were exposed to each parasitoid species and not exposed followed by different letters are significantly different by Dunn's test at $P < 0.05$

** Means (±SE) calculated are for each 10 egg/replicate

[a] These include the percentage of eggs from which neither parasitoids nor *H. halys* nymphs emerged

Table 2 Parasitism of unrinsed and rinsed *Halyomorpha halys* eggs by *Telenomus podisi* and *Trissolcus erugutus* females that had emerged from the first no-choice egg bioassays *

	No. exposed eggs	Treatments	Mean parasitized eggs**	Mean parasitoid emergence**	Mean BMSB nymphal emergence**	Mean egg mortality** [a]
Telenomus podisi	240	Unrinsed eggs	0.79±0.25a	0.54±0.2a	7±0.38a	2.37±0.44a
	180	Rinsed eggs	2.83±0.51b	0.77±0.24a	2.61±0.6b	4.55±0.4b
Trissolcus erugatus	250	Unrinsed eggs	0.76±0.26a	0.48±0.19a	5.72±0.48a	3.52±0.55a
	300	Rinsed eggs	3.33±0.62b	2.4±0.46b	1.23±0.37b	5.4±0.56b
Unexposed eggs	150	Unrinsed eggs	-	-	9.46±0.04a	0.53±0.03a
	130	Rinsed eggs	-	-	9.76±1.21a	0.23±0.03a

* Means (±SE) for each species/treatment pair within columns for *H. halys* eggs that were exposed to each parasitoid species and not exposed followed by different letters are significantly different by Dunn's test at $P < 0.05$

** Means (±SE) calculated are for each 10 egg/replicate

[a] These include the percentage of eggs from which neither parasitoids nor *H. halys* nymphs emerged

Highlights

- *Halyomorpha halys* populations are largely unaffected by native natural enemies in the USA
- We studied the importance of chemical compounds present on *H. halys* eggs to two North American scelionid wasps
- *Telenomus podisi* and *Trissolcus erugatus* were able to parasitize hexane-rinsed and frozen *H. halys* eggs
- Wasps' offspring from rinsed-frozen eggs parasitized unrinsed-unfrozen eggs for four or more generations
- Results suggest that artificially-selected wasps are potential candidates for *H. halys* biocontrol

7 ARTIGO 5

Hopkins' host selection principle revisited: learning and memory by *Telenomus podisi* and *Trissolcus erugatus* (Hymenoptera: Scelionidae) to parasitize *Halyomorpha halys* (Heteroptera: Pentatomidae) eggs*

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R. Tognon
Departamento de Fitossanidade,
PPG-Fitotecnia - UFRGS - Av.
Bento Gonçalves, 7712, 91540-000 Porto Alegre, RS, Brazil.
Phone: +55 51 33087414
Email: roberta.tognon@ufrgs.br

Hopkins’ host selection principle revisited: learning and memory by *Telenomus podisi* and *Trissolcus erugatus* (Hymenoptera: Scelionidae) to parasitize *Halyomorpha halys* (Heteroptera: Pentatomidae) eggs

R. Tognon[1], J.R. Aldrich[2,3], J. Sant’Ana[1] and F.G. Zalom

[1]Department of Crop Protection, PPG-Fitotecnia, Federal University of Rio Grande do Sul (UFRGS), Av. Bento Gonçalves, 7712, 91540-000 Porto Alegre, RS, Brazil.

[2]Jeffrey R. Aldrich Consulting LLC, 95061, Santa Cruz, CA, USA.

[3]Department of Entomology and Nematology, University of California, Davis, One Shields Avenue, 95616, Davis, California, USA.

Running head: Tognon et al.: Hopkins’ host selection principle revisited

ABSTRACT

Halyomorpha halys Stål (Heteroptera: Pentatomidae), the brown marmorated stink bug (BMSB), is a polyphagous fruit and vegetable pest from eastern Asia that is now invasive in North America and Europe. Parasitism of *H. halys* by endemic natural enemies in North America and Europe is typically low. The learning is one of the most important strategies to egg parasitoids find the host in a complex chemical environment. In this study, we evaluate the ability of two native parasitoids in North America, *Telenomus podisi* Ashmead and *Trissolcus erugatus* Johnson (Hymenoptera: Scelionidae) to learning and memorise chemicals from *H. halys* eggs (extracts and synthetic compounds previously identified in a later study) through chemotactic behavior and laboratory parasitism bioassays. Fresh *H. halys* eggs (1g) were covered with hexane in a glass vial for 5 min and the resulting extract was transferred to a new vial for using in bioassays. Both scelionids were reared in laboratory on *Euschistus conspersus* Uhler (Hemiptera: Pentatomidae) eggs, a native host in California. Parasitized colony eggs (*E. conspersus*) were coated with 5µL of BMSB egg extract (4.5 egg equivalent, EE) or synthetic mixture of three previously obtained aldehydes (hexadecanal, octadecanal and eicosanal) (0.1ng total). After emergence, the response of these experienced females was observed using a 'Y-tube' olfactometer. Moreover, the learning and memory, in subsequent generations, were also evaluated in parasitism assays. Inexperienced females (from *E. conspersus* without extract coating) were tested as a control. Results showed that females with no previous experience were not attracted either to egg extract or synthetic compounds and did not parasitize BMSB. Conversely, experienced parasitoids learned to respond to the volatiles. The positive response lasted at least 72h. Parasitism of both species in *H. halys* eggs was observed in three subsequent generations. Manipulation of natural enemies by learning is a relevant step for improving biological control.

KEY WORDS

Learning, invasive species, native natural enemies, pre-imaginal experience, behavioural manipulation

Egg parasitoids are known to use long and short-range cues as kairomone to find suitable hosts (Aldrich 1995; Borges et al. 1998; Bruni et al. 2000; Colazza et al. 2010), thus egg volatiles can play an important role to host recognition (Bin et al. 993; Renou et al. 1992; Tognon et al. 2014). In the 'variable response model' showed by Vet et al. (1995), wasps have innate responses to each series of chemical cues used for locating their target and such responses can be classified according to their potential effects, so the variability of wasp responses to each stimulus should be related to their potential benefits. The behavioural manipulation by experience acquired during development and/or the adult stage can modify innate parasitoid behavior (Hare et al. 1997; Turlings et al. 1993). A pioneer detailed study on parasitoid learning was conducted by Thorpe and Jones (1937) inducing the wasp *Venturia canescens* (Grav.) (Hymenoptera: Ichneumonidae) to parasitize an unusual host. The authors suggested that the female parasitoids preferred specific host species or some associate cues present in the immature stage development (pre-imaginal conditioning). A hypothesis that "a species which breeds on more than one host will demonstrate a preference for the host species on which it has become adapted" was primary postulated by Hopkins (1917), and it is known as Hopkins' host selection principle. Corbet (1985) put forth a more modern version of this hypothesis called the chemical legacy hypothesis, suggesting that actual traces of chemical cues inside or outside the host are carried over immature parasitoid into the adult stage, where they directly affect the sensitivity of the insect to these chemicals, as observed in *Trichogramma brassicae* (Hymenoptera: Trichogrammatidae) (Bjorksten and Hoffmann 1998) and *T. podisi* (Tognon et al. 2013, 2014). Thus, experiences during the adult stage affect the natural enemies' later host selection responses (Turlings et al. 1993; Vet and Groenewold 1990).

The learning ability by *T. podisi* Ashmead (Hymenoptera: Scelionidae) was first reported through the experience during the immature stage to lemongrass (*Cymbopogon citratus* (DC) Stapf.), a nonhost and innate repellent compound to this species (Tognon et al. 2013). Peri et al. (2006) have previously shown that chemical residues left on the substrate by adults of *Nezara viridula* (L.) (Hemiptera: Pentatomidae) act as indirect host-derived cues inducing arrestment of *Trissolcus basalis* (Wollaston) (Hymenoptera: Scelionidae) females, and that this memory persisted for at least 23 hours.

The target of our study, *H. halys*, the brown marmorated stink bug (BMSB), is an Asian species and newly invasive in North America and Europe. The BMSB is a polyphagous pest of fruit and seeds, including many that are of economic importance (Cissel et al.

2015; Leskey et al. 2012). Joseph et al. (2015) observed injuries greater than 90% on peach and apple under field conditions by *H. halys* feeding in Virginia, USA. The most concern detail for this invasive species is that it is becoming a serious global pest causing ecological imbalances (Zhu et al. 2012). It is associated to the lack of effective natural enemies where the BMSB is exotic (Abram et al. 2014; Cissel et al. 2015; Haye et al. 2015). In Asia, hymenopteran parasitoids of the genera *Trissolcus*, *Telenomus* (Scelionidae), *Ooencyrtus* (Encyrtidae) and *Anastatus* (Eupelmidae) are known to parasitize eggs of *H. halys*, however *Trissolcus japonicus* (Ashmead) and *Trissolcus cultratus* Mayr. (Hymenoptera: Scelionidae) (Chinese strain) are believed to be the most important organisms (Lee et al. 2013). Species of the same genera develop poorly or not at all on *H. halys* eggs in Europe (Haye et al. 2015) and North America, resulting in an exponential increase of BMSB in these areas (Abram et al. 2014). An important step was done to start understanding the low and/or absence of parasitism by native egg parasitoids in North America over *H. halys*. In one of our later study, we have identified chemical compounds on the external layer of BMSB eggs, a mixture of the following aldehydes: hexadecanal, octadecanal and eicosanal was elucidated. We observed that North American scelionid wasps avoid these substances from eggs and they also are not parasitized by them (Tognon et al. 2016). The current observation could be influenced by physiological and genetic characteristic provided by the isolation between the specific host and wasp populations (Arakaki et al. 1997), moreover, volatiles on BMSB egg masses are not a part of these parasitoids ecological context. On the other hand, we infer that recognition and subsequent parasitism by native parasitoids is modulated by specific chemical cues on the external egg surface to species with close relationship as verified to *E. conspersus*, a native phytophagous in California (Tognon et al. 2016). In this case, methyl (2*E*,4*Z*)-2,4-decadienoate, was the principal egg volatile of *E. conspersus* egg masses, which attracted parasitoid females either under laboratory or field assays. As this substance is known as the main component of the male-produced aggregation pheromone which elucidates kairomonal activity on scelionids, the attractiveness can be related to evaluative interaction between wasp/stink bug, whereas it is transferred from male to female by mating and impregnate on eggs during oviposition (Tognon et al. 2016). *Telenomus* sp. and *Trissolcus* sp. are known to use pheromone from heteropteran hosts to find their egg masses (Borges et al. 1998; Bruni et al. 2000; Tognon et al 2014), thus the *E. conspersus* attraction is most probably to be an evolutive interaction modulated by reliable cues that lead wasps to their target. In contrast, eggs of the brown marmorated stink bug, completely lack the sesquiterpenoid pheromone compounds, identified from males of this species (Khrimian et al. 2014), causing an absence of parasitism. Such observations are a concept applied on insect interaction between native/indigenous (probable avoidance), but is more usually accepted on association native enemies/native host (acceptance) (Cornell & Hawkins, 1993). This concept drive us to some questions: what is the influence of original host and its real contribution for wasp choice? Is it possible to manipulate the wasp choice? The behavioral manipulation of natural enemies is an opportunity for improving biological control efficiency in agroecosystems (Meiners & Peri, 2013). In this context, we evaluated chemical learning and memory by two native scelionid wasps in North America, *T. podisi* and *T. erugatus*, to compounds on the surface of *H. halys* eggs in laboratory bioassays.

Material and Methods

Stink Bug Colonies. A colony of *E. conspersus* nymphs and adults was obtained from Paramount Farming in Shafter, Kern County, CA, in December 2014 from collections made in the southern San Joaquin Valley during summer 2014. This colony was maintained in a Percival Model I-36LL growth chamber (Perry, IA, USA; 26±1°C, 65±10% RH, 16L: 8D photoperiod) at the University of California at Davis (UCD). Adults were maintained in 19 x 25 x 19-cm plastic cages with 13 x 13-cm hole in the top fitted with organza material. The insects were fed with organic green beans, sunflower seeds, cherry tomatoes, and provided water from cotton-stoppered shell vials (4.0 ml, 15-mm diameter, 45-mm height; J.G Finneran Associates, Inc., Vineland, NJ, USA). Each cage contained no more than 50 adults and food was replaced twice a week. Paper toweling lining cages served as an oviposition substrate. Eggs were collected from adult cages daily and maintained separately under the same conditions, as above, for colony maintenance or bioassays.

Halyomorpha halys nymphs and adults were collected in 2015 from Fremont Community Garden in Sacramento County, CA. The colony was maintained as described above for *E. conspersus*, except that pumpkin seeds and slices of apple, plum or cherries were also provided.

A colony of the spined soldier bug, *Podisus maculiventris* Say (Hemiptera: Pentatomidae), was established from adults collected at the arboretum of the University of California at Santa Cruz (UCSC) in Rescue® Stink Bug Traps (Sterling International, Inc., Spokane, WA, USA) baited with the synthetic aggregation pheromone of *P. maculiventris* (Aldrich et al. 1984). The *P. maculiventris* colony was maintained as for *E. conspersus,* however they were fed with larvae and pupae of *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) obtained from a commercial source (Rainbow Mealworms, Compton, CA, USA).

Parasitoid Colonies. *Trissolcus erugatus* and *T. podisi* were collected in the University of California at Davis (UCD) Student Farm and the UC Santa Cruz (UCSC) arboretum in fresh *P. maculiventris* eggs, by clipping masses onto leaves of garden plants and coastal live oak trees, respectively (Tognon et al. 2016). After 48 h, the sentinel eggs were removed from the field and returned to the laboratory where they were held in the respective laboratories at room temperature until parasitoids emerged. Colonies of both parasitoid species were maintained in a Percival Model I-36LL growth chamber (Perry, IA, USA) in the Zalom laboratory at UCD under the same conditions as for the pentatomids in this study, using *E. conspersus* eggs as hosts, and were provided with a drop of honey (~3 µL) every 3 days. *Trissolcus erugatus* and *T. podisi* were identified with keys of Talamas et al. (2015) and Johnson (1984), respectively.

Specimens of both egg parasitoids were sent to the Systematic Entomology Laboratory, USDA-ARS, Washington D.C., for identification; voucher specimens are deposited in the National Insect Collection, National Museum of Natural History, Smithsonian Institution. Voucher specimens for both the parasitoids and the pentatomid species have been deposited in the R. M. Bohart Museum of Entomology at UC Davis.

Experienced Parasitoid Females and Innate Behavior. Mated *H. halys* females were separated from males and kept in different cages with food, water and paper towelling for oviposition. Eggs (12-24 hours old) were removed from paper with forceps and then placed and weighed in a 2 mL borosilicate glass vials (Waters Corporations, Milford, MA, USA). Enough hexane (>99%, Sigma-Aldrich, St. Louis, MO, USA) was added to cover ~1g of eggs for 5 minutes and the resulting extract was placed in a new vial and used in bioassays.

Euschistus conspersus eggs (24 h old) were offered to *T. erugatus* and *T. podisi* to parasitism. After 24h, the masses were placed in a glass Petri dish, rinsed with 99% hexane for 5 min, air-dried and coated with 5 µL of *H. halys* egg extracts (5 µl = 4.5 eggs equivalent, EE) or synthetic aldehydes mixture (hexadecanal, octadecanal and eicosanal) (5µl = 0.1 ng total) previously identified (Tognon et al. 2016). This procedure was performed every 3 days for ca. 12 days, until 2 days before the emergence.

Olfactometry. Chemotactic responses of the experienced parasitoid females (24h old) as described above were evaluated in two-choice tests using a horizontally positioned Y-tube glass olfactometer (1.4-cm diameter), with a 16-cm basal arm, bifurcated at a 60° angle into two 19-cm arms.

Air flow was 0.8 L/min through an air pump connected to a flow meter and a humidifier. Before the experiment, each female was placed individually in a glass tube (5 ml), and fed with a drop of honey (3 µL). A single wasp was introduced into the Y-tube and allowed to choose between a piece of filter paper (1 x 2 cm, P5 Fisherbrand®, Fisher Scientific, Marshalltown, IA, USA) containing 5 µl of egg extracts of *H. halys* (4.5 EE) or a synthetic aldehydes blend (0.1 ng total), both in contrast with 5µL of hexane (control). Females from *E. conspersus* without contact with crude or synthetic *H. halys* eggs extracts (inexperienced insects), from both species, were also tested as control treatment. Furthermore, memory was tested by repeated bioassays every 24 h, for 4 days, in the experienced females with synthetic blend of *H. halys* eggs.

All bioassays were conducted in an acclimated room (24 ± 2°C and 60 ± 10% RH) during the photophase period with a fluorescent bulb (9 W, luminance = 290 lux) above the bioassay arena surrounded by a paper wall to minimise possible external cues. After three insects were assayed, the odor sources were switched to the opposite side to avoid positional bias. Glassware was rinsed for each of the six replicates with fragrance-free liquid soap followed by distilled water, and dried in a convection oven at 100^{o}C. Each insect was given 10 min to make a choice of arms in the olfactometer. Parasitoids that moved at least 3 cm into one branch arm and remained there for at least 60 s, were recorded as responsive. If no choice was made in 10 min, the insect considered non-responsive, excluded from statistical analysis, and the assay was concluded. At least 40 replicates were performed for each test.

Parasitism tests. *Halyomorpha halys* eggs were offered to experienced females that developed either in the presence of egg extracts or synthetic compounds. Inexperienced females (innate behavior) were tested as a control. Fresh clusters of *H. halys* were collected from the laboratory colony. An egg mass with 10 eggs was glued onto filter paper (1 x 1.5 cm) using double-sided tape, and placed in a glass tube (7.5 x 1.3 cm) with a female (24-h old) of *T. podisi* or *T. erugatus* for 24 hours. The tube was provided with drop of honey as food for the parasitoids, and sealed using parafilm. Females were then removed, and parasitoid emergence was recorded for a period of 3-4 weeks. New fresh *H. halys* eggs were offered to the first and subsequent generations of both parasitoids,

Statistical analyses. The choice on the olfactometer was analysed by χ^2-tests and Kruskal-Wallis H-test. Means of parasitized eggs, parasitoid or stink bug emergence and egg mortality were compared within treatments by Kruskal-Wallis, all tested 95% significance level, with BioEstat® 5.0 (Ayres et al. 2007).

Results

Olfactometry. Inexperienced *T. erugatus* females' choice was significantly more often the control treatment (62.3%) than the egg volatile treatment (30.9%). However, subsequent experiments the experienced females changed their behavior being significantly more responsive to egg volatiles treatment (61.4%) than to the hexane control treatment (36.4%) ($\chi2 = 5.628$; fd = 1; $P = 0.0310$) (Figure 1).

A similar result was observed to *T. podisi*. Inexperienced parasitoids chose the control (69%) more than the extract (23.8%) ($\chi2 = 18.513$; fd = 1; $P = 0.0001$); while experienced wasps preferred the egg extract treatment (60.5%) over the control (27.9%) ($\chi2 = 10.316$; fd = 1; $P = 0.0029$) (Figure 1).

Both *T. erugatus* (Figure 2) and *T. podisi* (Figure 3) females without experience with treatment aldehydes were repelled ($P > 0.05$). Conversely, experienced females learned to respond to the synthetic aldehyde mixture, and retained this memory for at least 72 hours ($P < 0.05$); afterward, their behavior was similar to that of inexperienced wasps ($P > 0.05$).

Parasitism Tests. The parasitism of experienced *T. erugatus* toward egg extracts was not significantly different from that of experienced females toward the synthetic aldehyde mixture ($P > 0.05$) whereas, for *T. podisi*, females experienced toward egg extracts were 7.5% statistically different to 19.3% ($P < 0.05$). Nevertheless, in both species, successful parasitoid emergence was only observed from females previously exposed to the synthetic aldehydes (Table 1). In addition, nymphal hatching was higher and egg mortality was lower ($P < 0.01$) when both parasitoid species were inexperienced or in unexposed eggs to parasitism, compared to the experienced ones (Table 1).

Trissolcus erugatus females that had experience with the synthetic aldehydes were able to parasitize significantly more eggs until 24 h old when compared with 72 h, but it was not different of 48 h (Table 2). The parasitism rate decreased after 24 h old. For *T. erugatus*, parasitoid emergence was observed only to treatment in which wasps were 24 and 48 h old. *Telenomus podisi* females were able to parasitize host eggs until 96 h after emergence; however, after 48 h, the parasitism rate also decreased significantly. No parasitoid emergence was observed in females with 96 h old (Table 2). In both tests, hatched nymphs were lower and egg mortality higher in the youngest wasps (Table 2). After the first generation in BMSB, *T. erugatus* decreased their parasitism and emergence success ($P > 0.05$). The average of hatched nymphs and egg mortality was similar in all steps. On the other hand, parasitism of *T. podisi* had no difference between the first and second generation, but decreased in the third generation compared to the first. Consequently, nymph averages was low on the first and second treatment but high on the third one. The BMSB egg mortality decreased after the first generation only when eggs were exposed to *T. podisi* (Table 3).

Discussion

Inexperienced females of *T. podisi* and *T. erugatus* did not recognize *H. halys* eggs as a potential host. Indeed, the development of endemic scelionid species on *H. halys* eggs in Europe was low or absent (Haye et al. 2015). Our previous work showed that scelionid wasps avoided odorants from the brown marmorated stink bug egg masses, likely because BMSB eggs contain repellent compounds to protect them for natural enemies (Tognon et al. 2016). Similar chemical defences were also described for many insect species (Gillot 2002; Hinton 1981), including heteropterans as observed in *Caenocoris nerii* (Germar) (Lygaeidae) eggs (von Eaw et al. 1971) and *Murgantia histrionica* (Hahn) (Pentatomidae) adults (Aliabadi et al. 2002).

However, experienced *T. erugatus* and *T. podisi* females learned to respond to crude extract and synthetic aldehydes mixture through an experience acquired during immature stage. Response of parasitoids to semiochemicals is determined and influenced by genetic and physiological factors, as well as, by environmental parameters and experience (Vet et al. 1995). A considerable degree of plasticity, explained as the ability of one organism to change its physiology in response to environment conditions, manifested by an individual insect, may have a significant impact on evolutionary changes in host use (Wäschke et al. 2013).

When a parasitoid lays eggs on a nonhost species, the offspring may display an increased tolerance or even preference for the novel host, starting the shift (Jaenike & Papaj, 1992). Our findings are reminiscent and pertinent to Hopkins' host selection principle (Hopkins, 1917). In this study, we could observe this aspect with the experienced females responding for crude extract, synthetic aldehydes mixture and subsequently parasitizing the egg masses of the previous unknown host. This result corroborates those observed by Tognon et al. (2013) whose reported that *T. podisi* was attractive to essential oil of lemongrass, a nonhost odor and primary repellent to this species, after a pre-imaginal conditioning. Another clear example of this theory is involving methyl (2*E*,4*Z*)-2,4-decadienoate identified on *E. conspersus* egg masses (Tognon et al. 2016). A male-specific aggregation pheromone (Aldrich et al. 1991), transferred from male to females by mating, stays impregnated on layer eggs and later, after a parasitoid emergence from this host, it is used for locating new eggs as a reliable kairomone (Tognon et al. 2016).

Although, learning during immature stage led *T. podisi* and *T. erugatus* to parasitize BMSB eggs, until 72 and 96 hours old, respectively. It was already known that *T. podisi* is more likely to parasitize the host in which it development occurred, preserving a chemical legacy from the odor that its larvae were exposed (Tognon et al. 2014). Both species exhibit a long-term memory as classified by Margulies et al. (2005) to *Drosophila* and proven by van den Berg et al. (2011) to parasitoid wasps. The memory at 4-5 days observed in our work is considered long for scelionid since their life span is short under natural environmental. In an ecological context, this memory time would enable them to have enough time to find a suitable host easer, increasing their biological fitness and parasitism success, since the females' peak search start on the first and second alive days (Yeargan 1982).

Parasitoids memory ability registered here is probably related to the brain lobes formation during larval stage. Holometabola insects have a series of transformations resulting in changes in the nervous system (Barron and Corbet, 1999), however in *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae), parts of the mushroom body responsible for the storage of chemosensory information, remain intact during metamorphosis, potentially allowing memory to be maintained from larvae to adults (Armstrong et al. 1998).

It was already observed in *Drosophila* that neurons development of the three-lobed on mushroom-body occurs sequentially: the γ lobe is formed in larval stage, the α / β starts at the beginning of the formation of the pupa and neurogenesis of α '/ β' lobes develop just before pupation. During this phase of the γ lobe neurons suffer histolysis, disappearing, while the neurons α '/ β' and α / β keeps intact its projections after metamorphosis (Lee et al. 1999). Thus, we can infer that either *T. erugatus* or *T. podisi* physiologically lead information to adult stage though the metamorphosis. Furthermore, parasitoids overcome a chemical barrier in BMSB for parasitizing its host for three generations, but it decreased in the second to third one. The results were probably driven by chemical legacy (Corbet 1985), which may result in an evolutionary adaptation (Dukas 2008).

Telenomus podisi and *T. erugatus* are potential natural enemies of *H. halys* population in North America. Exploring the learn ability of these insects may be an alternative to improve its efficiency to be used in biological control by augmentative releases or conservative biological control programs. The behavioural manipulation with an arrestment effect on a parasitoid can be a potential solution to attract and reward strategies (Borges and Aldrich 1994). This hypothesis is supported by Hare et al. (1997) whose demonstrated that *Aphytis melinus* DeBach (Hymenoptera: Aphelinidae), a parasitoid of the California red scale *Aonidiella aurantii* (Maskell) (Hemiptera: Dispididae) exposed to a host synthetic kairomone, prior to being released in the field, improves its parasitoid activity in *A. aurantii,* a usually non-preferred host. Therefore, semiochemicals has a potential to be applied in mass rearing systems "teaching" parasitoids to find alternative hosts, directing the wasp for a target pest (Colazza et al. 2010) especially for exotic species with no suitable natural enemies. Thus, we believe our study is a relevant step to understand how to manage native parasitoid behavior to improve biological control programs.

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Figure legends

Fig. 1. First choice of *Trissolcus erugatus* and *Telenomus podisi* females 24h old (from *Euschistus conspersus*) without (innate behavior) and with experience to odor of egg extracts of *H. halys*, tested in Y-tube olfactometer to volatiles of *H. halys* eggs and hexane (control). Numbers in parentheses represent the number of responsive insects to the treatments. Value followed by an asterisk differ significantly (χ^2, $P < 0.05$) of control within each treatment (inexperienced or experienced females).

Fig. 2. First choice of *Trissolcus erugatus* from *Euschistus conspersus* inexperienced (innate behavior) and experienced females to a mixture of synthetic aldehydes (0.1ng total) in a ‘Y-tube’ olfactometer. Asterisks indicate significant differences for paired comparisons between females’ innate behavior (inexperienced) and learning behavior (experienced) by Chi-square test ($P < 0.05$), to each time. Bars with different letters indicate differences for multiple comparisons by Kruskal-Wallis ($P < 0.05$) among times within each group tested (experienced and inexperience insects). Numbers represent the total of responsive insects to synthetic mixture.

Fig. 3. First choice of *Telenomus podisi* from *Euschistus conspersus* inexperienced (innate behavior) and experienced females to a mixture of synthetic aldehydes (0.1ng total) in a ‘Y-tube’ olfactometer. Asterisks indicate significant differences for paired comparisons between females’ innate behavior (inexperienced) and learning behavior (experienced) by Chi-square test ($P < 0.05$), to each time. Bars with different letters indicate differences for multiple comparisons by Kruskal-Wallis ($P < 0.05$) among times within each group tested (experienced and inexperience insects). Numbers represent the total of responsive insects to synthetic mixture.

Fig 1

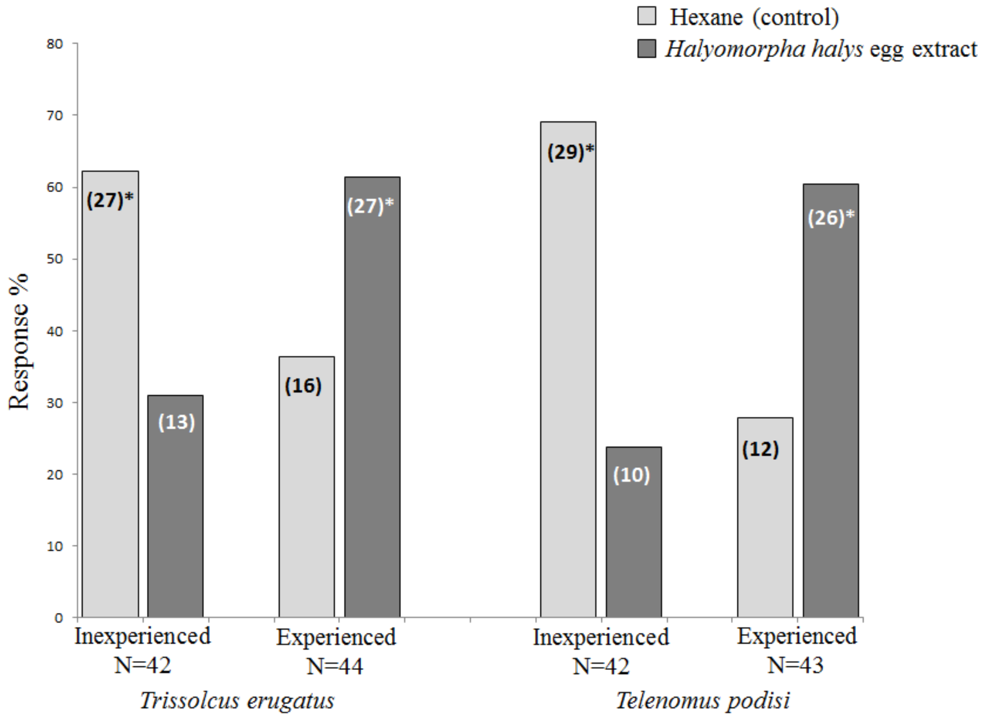
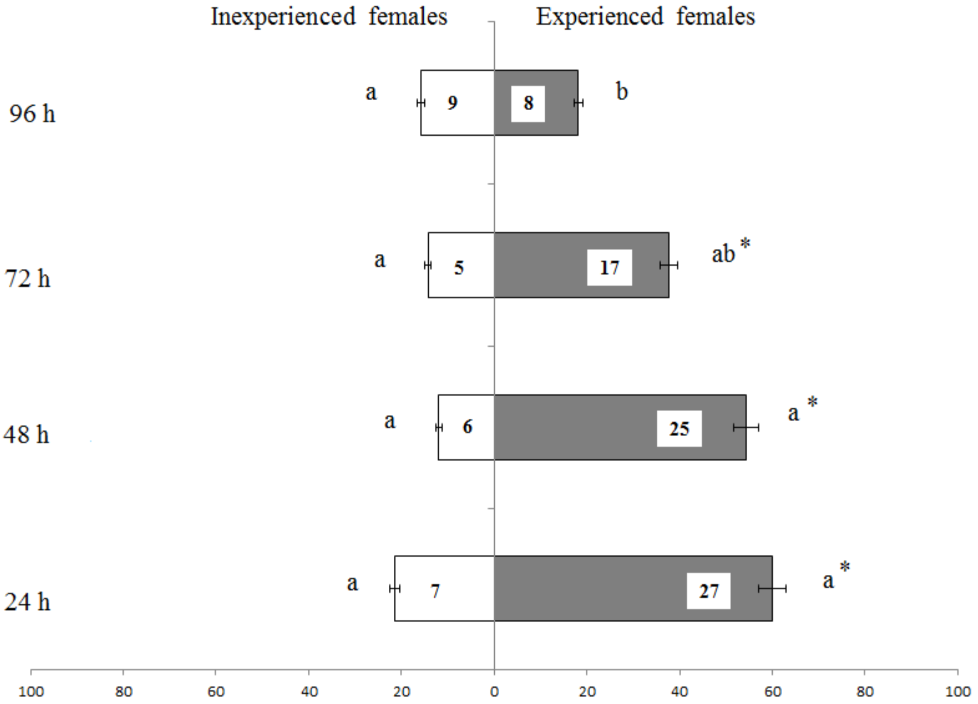

Hexane (control)
Halyomorpha halys egg extract
Response %
0
10
20
30
40
50
60
70
80
(27)*
(13)
(16)
(27)*
(29)*
(10)
(12)
(26)*
Inexperienced N=42
Experienced N=44
Trissolcus erugatus
Inexperienced N=42
Experienced N=43
Telenomus podisi

Fig 2


Inexperienced females
Experienced females
96 h
a
9
8
b
72 h
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ab *
48 h
a
6
25
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24 h
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100
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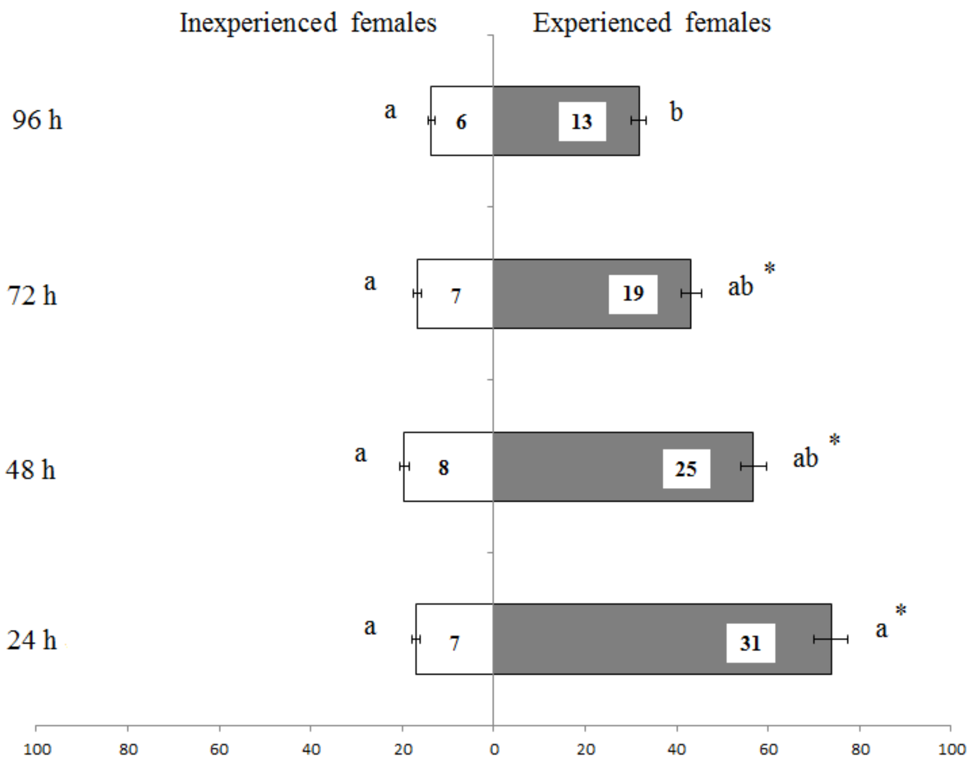
Fig 3


Inexperienced females
Experienced females
96 h
72 h
48 h
24 h
a
6
13
b
a
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19
ab *
a
8
25
ab *
a
7
31
a *
100
80
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20
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100

Tables

Table 1 Fresh *Halyomorpha halys* eggs offered to *Trissolcus erugatus* and *Telenomus podisi* in different treatment 1) experienced females on *H. halys* egg extracts, 2) experienced females on synthetic aldehydes, 3) females without previous exposed to chemicals (innate behavior), 4) *H. halys* eggs unexposed to parasitism.

Parasitoid species	Treatments	No. exposed eggs	Mean ± SE of parasitized eggs**	Mean ± SE of parasitoid emergence**	Mean ± SE of hatched nymphs**	Mean ± SE of egg mortality**^{a}
Trissolcus erugatus						
	1	250	2.24±0.36a	0	2.72±0.4a	5.04±0.42a
	2	290	3.13±0.38a	2.62±0.28	4.17±0.42b	2.62±0.35b
	3	240	0	0	9.5±0.18c	0.5±0.18c
	4	220	-	-	9.77±0.11c	0.22±0.11c
Telenomus podisi						
	1	200	0.75±0.12a	0	3.2±0.52a	6.05±0.5a
	2	300	1.93±0.34b	0.86±0.11	2.06±0.33a	6±0.43a
	3	270	0	0	9.81±0.09b	0.18±0.09b
	4	180	-	-	9.66±0.19b	0.33±0.19b

* Within the column, means (±SE) followed by different letters are significantly different ($P<0.05$).

** Means (±SE) calculated are for each 10 egg/replicate

[a] The proportion of eggs from which nothing emerged, probably caused by the oviposition attempt of *Trissolcus erugatus* or *Telenomus podisi* but no success development.

Table 2 Parasitism of *Halyomorpha halys* eggs by *Trissolcus erugatus* and *Telenomus podisi* experienced to synthetic aldehydes of different age classes, and outcomes

Parasitoid species	Parasitoid females age	No. exposed eggs	Mean ± SE of parasitized eggs**	Mean ± SE of parasitoid emergence**	Mean ± SE of hatched nymphs**	Mean ± SE of egg mortality** [a]
Trissolcus erugatus						
	24h old	290	3.13±0.38a	2.62±0.28a	4.17±0.42a	2.68±0.35a
	48h old	260	2.19±0.32ab	1.88±0.28a	5.34±0.39ab	2.46±0.38a
	72h old	180	1.44±0.2b	-	7.27±0.3bc	1.27±0.33ab
	96h old	180	0	-	8.94±0.46c	1.05±0.46b
Telenomus podisi						
	24h old	300	1.93±0.33a	0.86±0.1a	2.06±0.33a	6±0.43a
	48h old	250	2.04±0.24a	1.8±0.23b	3.68±0.41a	4.28±0.51ab
	72h old	240	0.41±0.14b	0.41±0.14a	7.5±0.62b	2.08±0.66bc
	96h old	220	0.5±0.17b	0	8.13±0.44b	1.36±0.48c

* Within the column, means (±SE) followed by different letters are significantly different ($P<0.05$).

** Means (±SE) calculated are for each 10 egg/replicate

[a] The proportion of eggs from which nothing emerged, probably caused by the oviposition attempt of *Trissolcus erugatus* or *Telenomus podisi* but no success development.

Table 3 Parasitism of *Halyomorpha halys* eggs by *Trissolcus erugatus* and *Telenomus podisi* in the subsequent generations, and outcomes

Parasitoid species	Parasitoid generation	No. exposed eggs	Mean ± SE of parasitized eggs*	Mean ± SE of parasitoid emergence*	Mean ± SE of hatched nymphs*	Mean ± SE of egg mortality**[a]
Trissolcus erugatus						
	1st	290	3.13±0.38a	2.62±0.28a	4.17±0.42a	2.68±0.35a
	2nd	160	1.12±0.25b	0.93±0.21b	4.75±0.5a	4.12±0.56a
	3rd	160	1.43±0.31b	0.56±0.15b	5.68±0.7a	2.87±0.68a
Telenomus podisi						
	1st	300	1.93±0.34ab	0.86±0.11a	2.06±0.33a	6±0.43a
	2nd	240	2.83±0.49a	2.66±0.47b	3.29±0.44a	3.87±0.49b
	3rd	150	0.73±0.24b	0.53±0.23a	5.93±0.74b	3.33±0.69b

* Within the column, means (±SE) followed by different letters are significantly different ($P<0.05$).

** Means (±SE) calculated are for each 10 egg/replicate

[a] The proportion of eggs from which nothing emerged, probably caused by the oviposition attempt of *Trissolcus erugatus* or *Telenomus podisi* but no success development.

8 CONSIDERAÇÕES FINAIS

Neste estudo constatou-se que o comportamento de busca e as taxas de parasitismo podem ser alterados através de processos de aprendizagem química de parasitoides, os quais podem ser desencadeados tanto pela exposição a compostos cairomonais (extratos ou sintéticos) como pelo tipo de hospedeiro de origem. Este estudo abrirá novas ideias e oportunidades na aplicabilidade e manipulação de insetos benéficos tanto em técnicas de controle biológico conservativo, como em criações massais destinadas ao controle inundativo. No primeiro caso, os cairomônios poderiam ser aplicados no campo com a função de atrair parasitoides, manipulando seu comportamento para sincronizar sua população com a do herbívoro. De forma complementar, a experiência com o odor de um novo hospedeiro poderia proporcionar, de forma mais rápida e eficaz, o encontro entre o parasitoide e o hospedeiro alvo, maximizando as chances de parasitismo.

Esta linha de pesquisa tem caráter inovador e pode ser uma ferramenta útil em programas de controle biológico de pragas. No entanto, tal como todas as inovações, a manipulação comportamental de parasitoides deve ser avaliada de forma criteriosa para que possa realmente proporcionar um incremento no uso de inimigos naturais em agroecossistemas.