CONDICIONAMENTO PRÉ-IMAGINAL NO COMPORTAMENTO DE PREDAÇÃO, CUSTO METABÓLICO E PERFIL QUÍMICO DA CERA DAS JOANINHAS *Cryptolaemus montrouzieri* MULSANT E *Tenuisvalvae notata* (MULSANT) (COLEOPTERA: COCCINELLIDAE)

por

NATALY DE LA PAVA SUÁREZ

(Sob Orientação da Professora Christian Sherley Araújo da Silva Torres - UFRPE)

RESUMO

A liberação de inimigos naturais (parasitoides e predadoras) é uma das táticas do manejo integrado de pragas. Dentre os predadores, destacam-se as joaninhas *Cryptolaemus montrouzieri* e *Tenuisvalve notata*, ambas predadoras de cochonilhas farinhentas (Pseudococcidae). A produção massal destes predadores padroniza as condições de criação e gera uma pressão de seleção que pode alterar o desempenho do predador após várias gerações em insetários. Diante disso, avaliou-se se as joaninhas *T. notata* e *C. montrouzieri* apresentam preferência alimentar e resposta olfativa pela presa na qual foram criadas, se existe custo metabólico associado na produção de cera na fase larval destes predadores, e se existe efeito da composição química da cera na interação predador-presa. As presas utilizadas foram *Ferrisia dasylirii* e P*lanococcus citri*. Observou-se que as joaninhas, responderam de forma semelhante aos voláteis liberados pelas espécies de presas oferecidas. Além disso, não houve mudanças no comportamento do predador em relação às pistas olfativas das presas. Ambas espécies de joaninhas consumiram mais *P. citri* em testes de laboratório e semi-campo, independentemente da presa usada na criação, indicando não haver

condicionamento pré-imaginal do predador. Em relação à cera, existe um custo metabólico associado à produção da mesma, com uma redução significativa no peso corporal do adulto, fecundidade e viabilidade dos ovos quando a cera foi removida durante a fase larval. Por fim, a interação predador-presa não condiciona a composição química de cera das joaninhas, visto que diferem dos perfis químicos da cera da presa.

PALAVRAS-CHAVE: Condicionamento pre-imaginal, Pseudococcidae, camuflagem físico-química, controle biológico.

PRE-IMAGINAL CONDITIONING ON THE PREDATION BEHAVIOR, METABOLIC COST AND WAX CHEMICAL PROFILE OF THE LADY BEETLES *Cryptolaemus montrouzieri* MULSANT E *Tenuisvalvae notata* (MULSANT) (COLEOPTERA COCCINELLIDAE)

by

NATALY DE LA PAVA SUÁREZ

(Under the direction of Professor Christian Sherley Araújo da Silva Torres - UFRPE)

ABSTRACT

Releasing natural enemies (parasitoids and predators) is a practice of integrated pest management. Among the predators, the lady beetles *Cryptolaemus montrouzieri* e *Tenuisvalve notata* are outstanding predators of mealybugs (Pseudococcidae). Large production of predators under standard rearing conditions may create selection pressure and alter their performance after many generations in insectaries. Therefore, we evaluated: if the lady beetles *T. notata* and *C. montrouzieri* show food preference and olfactory response to prey they were reared; if there is a metabolic cost associated with wax production in larvae of these predators; and if there is any effect of the wax chemical composition in the predator-prey interaction. Prey used were *Ferrisia dasylirii* and P*lanococcus citri*. We found that the lady beetles responded similarly to volatiles released by either prey species offered. Also, there were no changes in predator behavior to prey olfactory cues. Both lady beetle species consumed more *P. citri* in laboratory and semi-field tests, regardless of rearing prey, indicating no pre-imaginal conditioning. Regarding the wax, there was a metabolic cost associated with its production, with a significant reduction in adult

predator body weight, fecundity, and egg viability when the wax was removed during the larval stage. Finally, the predator-prey interaction does not condition the chemical composition of wax in lady beetles, as there is a difference in the chemical profiles of wax from the prey.

KEY WORDS: Pre-imaginal conditioning, Pseudococcidae, physical-chemical camouflage, biological control.

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CAPÍTULO 1

INTRODUÇÃO

Uso de Inimigos naturais no controle de pragas

O controle biológico envolve o uso de qualquer organismo vivo que suprime ou diminui as populações de espécies que dada sua alta densidade causam danos aos seres humanos (pragas) (Van Driesche *et al.* 2009). Estes organismos são chamados inimigos naturais pois geram mudanças permanentes diretas ou indiretas nas teias alimentares que cercam as pragas (DeBach 1964). Dentro dos inimigos naturais de insetos, os mais conhecidos e usados no controle biologico são os insetos entomófagos (predadores e parasitoides), organismos que requerem alimentar-se de outros insetos para completar seu crescimento pelo menos numa fase de desenvolvimento (Montgomery 2011). Dependendo de como estes organismos são usados no campo, podem ser categorizados em controle biológico aplicado ou natural por conservação. No aplicado, há o controle biológico clássico, quando o inimigo natural é uma espécie exótica introduzida para o controle de outro organismo que invadiu novas áreas e por tanto necessita de fatores que regulem suas populações (Fischbein & Corley 2022).). Além desse, também há o controle biológico aumentativo, que permite aumentar as populações de inimigos naturais em campo através da liberação massal dos mesmos (Parra *et al*. 2002). Predadores e parasitoides são os organismos mais comuns nos quais se desenvolve este tipo de práticas, isso pela habilidade que tem para detetar pistas no ambiente para localizar suas presas e hospedeiros (Sethuraman *et al*. 2020). Entre os predadores as famílias Coccinellidae e Chrysopidae (Van Driesche *et al.* 2009). No controle biológico conservacionista, envolve todas as ações para proteger, manter e aumentar as

populações de inimigos naturais já existentes nas áreas, sem a introdução de organismos exóticos (Eilenberg *et al.* 2001).

O primeiro caso de controle biológico bem-sucedido envolveu a introdução da joaninha *Rodolia cardinalis* (Mulsant) para controlar a cochonilhas, conhecida como pulgão-branco dos cítricos, *Icerya purchasi (*Maskell), em plantas de *Citrus* na Califórnia durante o final da década de 1880 (Caltagirone & Doutt 1989). Outras espécies de joaninhas predadoras importantes reportadas no controle biológico são: *Cryptolaemus montrouzieri* (Mulsant), introduzida para o controle de *Planococcus citri* (Risso) na Califórnia, e usada em mais de 40 países nas regiões temperadas e tropicais para o controle de cochonilhas (Maes *et al.* 2015); *Hyperaspis pantherina* (Fürsch), espécie africana introduzida na ilha de Santa Helena para o controle da cochonilha *Orthezia insignis* (Browne) (Fowler 2004); e *Coleomegilla maculata* (De Geer) usada nos Estados Unidos como um importante predador de ovos de várias Lepidopteras e do besouro praga da praga da batata *Leptinotarsa decemlineata* (Say) (Hazzard *et al*. 1991).

No entanto, os programas de criações massais de inimigos naturais e as introduções em campo podem fracassar se não se realiza uma correta identificação do organismo a multiplicar ou se desconhece a biologia e ecologia do mesmo (Parra *et al.* 2002). Alguns fatores reportados que levam a falhas no controle são: predação intra-guilda, competição por nicho e recursos com espécies nativas, falta de hospedeiros alternativos, falta de sincronia nos ciclos biológicos do predador e a praga, entre outros (Hajek *et al.* 2016). Isto demostra que é necessário antes da produção e liberação de inimigos naturais, conhecer a biologia do hospedeiro ou presa, e os fatores que participam e modulam a interação dos mesmos no ambiente.

Coccinellidae

Esta é uma família da ordem Coleoptera, caracterizada pelos hábitos predatórios da maioria das suas espécies e pela aparência vistosa dos adultos, conhecidos comumente como joaninhas (Majerus 1994, Giorgi *et al.* 2009). Esta família conta com 6000 a 7000 espécies descritas, distribuídas em 360 gêneros e 42 tribus (Vandenberg 2002, Nedvěd & Kovář 2012, Soares *et al*. 2023), das quais aproximadamente 2000 espécies estão registradas na região Neotropical (Almeida & Ribeiro-Costa 2009; Soares *et al*. 2023). Embora as espécies de Cocinellidae sejam conhecidas pelos seus hábitos predatórios (entomófagas), nem todas as espécies de joaninhas apresentam este tipo de alimentação, sendo algumas espécies classificadas como micófagas, e ainda outras como fitófagas (Majerus 1994, Giorgi *et al.* 2009). As joaninhas entomófagas, tanto na fase de larva como adulta possuem uma alta voracidade e atividade na busca das suas presas, por isso são muito utilizadas para controlar populações de pulgões, moscas-brancas, psilídeos, tripes, ácaros, cochonilhas, ovos de alguns lepidópteros, e lagartas neonatas (Hodek 1973, Gordon 1985, Majerus & Kearns 1989, Hodek *et al*. 2012).

A preferência alimentar das espécies desta família pode caracterizar-se dependendo da subfamilia que pertencem, assim: as espécies de Coccinellinae são predadoras predominantemente de pulgões e psilídeos; Chilocorinae de pulgões e cochonilhas; Ortaliinae de cigarrinhas, psilídeos e formigas; Sticholotidinae de pulgões e cochonilhas; Scymninae de ácaros, pulgões e cochonilhas (Giorgi *et al.* 2009).

Pelos seus hábitos alimentares e sua eficiência no controle de outros artrópodes, as joaninhas têm sido usadas ativamente em programas de controle biológico, através de liberações massivas de populações em campo (Mason *et al.* 2023). Por isso não é estranho que o primeiro caso de controle biológico de sucesso foi com a introdução da joaninha *R. cardinalis* (Mulsant) e

vários outros programas de controle biológico clássico de sucesso envolveram espécies de joaninhas joaninhas.A partir dessas liberações bem sucedidas várias espécies de joaninhas têm sido adaptadas e criadas de forma massal, como uma estratégia para regular as populações de insetos-praga, normalmente invasoras de novas áreas (Iperti 1999, Obrycki *et al*. 2009). Alguns exemplos dessas espécies são: *Cryptognatha nodiceps* (Marshall), *Hyperaspis pantherina* (Fürsch), *Rhyzobius lophanthae* (Blaisdell), *Chilocorus nigritus* (Fabricius) e *Chilocorus bipustulatus* (Linnaeus), para o controle de cochonilhas. *Harmonia axyridis* (Pallas), *Coccinella septempunctata* (Linnaeus) e *Diomus pumilio* (Weise) para o controle de *Sternorrhyncha* (Obrycki & Kring 1998, Fidelis *et al*. 2023).

Na América do Sul, e particularmente no Brasil, a espécie *Cryptolaemus montrouzieri* Mulsant foi importada do Chile para o controle da cochonilha-branca dos citros, *Planococcus citri* (Hemiptera: Pseudococcidae) (Gravena 2003). Além das espécies exóticas, existem outros potenciais controladores nativos que fazem parte do controle biológico conservacionista, onde através de estratégias de manejo de paisagem e do controle biológico aumentativo se promove o incremento e estabelecimento de populações de inimigos naturais nativos das áreas de infestação da praga. Um exemplo disso é a joaninha *Tenuisvalvae notata* (Mulsant), nativa de América do Sul e importante predador de cochonilhas, dada sua alta voracidade tanto na fase larval quanto adulta (Barbosa *et al*. 2014).

Joaninhas Coccidófagas

São classificados de coccidófagos todos os coccinelídeos que se alimentam de cochonilhas (Hemiptera: Sternorrhyncha) (Fisher *et al.* 1999). Esse tipo de organismo têm um papel importante nas estratégias de controle biológico, dado que, diferentemente dos afidófagos

(predadores de afídeos), apresenta um menor tempo de desenvolvimento, o qual coincide com o tempo de desenvolvimento da presa (Dixon *et al.* 1997, Milonas *et al.* 2015). Com relação à eficiência no controle, as espécies coccidófagas também são consideradas melhores que outros predadores desta familia, porque são mais vorazes e apresentam uma alimentação constante sem grandes períodos de inatividade (Dixon & Dixon 2000). Portanto, apesar de existirem um maior número de espécies afidófagas, são os coccidófagos historicamente usados na liberação de inimigos naturais contra pragas Sternorrhyncha ao redor do mundo (Fisher *et al.* 1999, Fischbein & Corley 2022).

Entre as joaninhas com preferência alimentar por cochonilhas, destacam-se no Brasil as espécies *C. montrouzieri* e *T. notata,* a primeira delas amplamente usada em programas de controle biológico clássico ao redor do mundo (Jiang *et al.* 2009) e a segunda espécie tem distribuição entre o planalto da Colômbia e o norte de Paraguai, com um alto potencial na implementação de controle biológico por conservação em áreas de ocorrência natural (Barbosa *et al.* 2014). Ambas espécies são coccidófagas, caracterizadas por apresentarem nas fases de larva e pupa uma cobertura de secreção cerosa branca, visualmente parecida à cera produzida pelas presas que consomem (Figura 1a-b [Material Suplementar]). De acordo com alguns autores, esta é uma estratégia de defesa contra seus próprios predadores e parasitoides associados às presas, além de ser uma estratégia de camuflagem para facilitar a entrada na colônia das presas de forma desapercebida, "lobo na pele do cordeiro" (Figura 2 [Material Suplementar]). (Pope 1979, Völkl & Vohland 1996, Majerus *et al.* 2007, Pérez-Rodríguez & Messelink 2023).

Cryptolaemus montrouzieri **Mulsant**

Esta joaninha é nativa da Austrália, conhecida popularmente como "destruidora de cochonilhas", é usada para controle da cochonilha-branca-dos-citros, *Planococcus citri* (Risso), da cochonilha-de-listra *Ferrisia virgata* (Cockerell) em plantas ornamentais no Egito (Attia & El-Arnouty 2007) e da cochonilha-rosada *Maconellicoccus hirsutus* (Green) na Índia (Mani & Krishnamoorthy 2008). Esta espécie se destaca pela alta capacidade predatória, tanto na fase larval como nos adultos. Para as larvas completarem o desenvolvimento podem consumir de 2000 a 3000 ovos, 300 ninfas de cochonilhas de *Planococcus citri* (Risso) ou 30 cochonilhas adultas (Santa-Cecília & Souza 2005, Mani *et al*. 2008). Os adultos podem chegar a consumir uma média diária de 33 - 37,5 ninfas (Al Khateeb & Raie 2002). É diferente da maioria das joaninhas comuns, *C. montrouzieri* apresenta uma coloração escura nos élitros, onde a única diferença observável entre macho e fêmea é a cor das tíbias das pernas anteriores, onde o macho apresenta coloração amarela a avermelhada, enquanto a fêmea apresenta os três pares de pernas de cor preta (Pang & Gordon 1986).

Tenuisvalvae notata (**Mulsant)**

Este coccinelídeo é nativo da América do Sul, com distribuição nos planaltos colombianos onde foi reportada predando *Phenacoccus herreni* (Cox & Williams), enquanto no sul do Brasil e Paraguai ela tem como presa preferencial *P. manihoti* (Loehr *et al.* 1990, Sullivan *et al.* 1991). No nordeste do Brasil, especificamente no semiárido de Pernambuco foi registrada predando as cochonilhas *Ferrisia dasyrilii* (Cockerell) e *Phenacoccus solenopsis* (Tinsley) (Barbosa *et al*. 2014). Esta espécie foi introduzida na África nos anos 80, como uma estratégia de controle da

cochonilha *Phenacoccus manihoti* (Matile-Ferrero) junto ao parasitoide *Apoanagyrus lopezi* (DeSantis) (Herren & Neuenschawabder 1991, Chakupurakal *et al*. 1994).

No Brasil, apesar de não ser usada em liberações massais, *T. notata* é uma espécie com alto potencial como controlador de cochonilhas (Pseudococcidae) uma vez estabelecida em campo, dado que uma fêmea adulta desta joaninha consome, em média, 157,9 ninfas neonatas, ou 3,6 ninfas de terceiro instar ou ainda 2,2 fêmeas de *F. dasylirii* por dia (Barbosa *et al*. 2014).

Diferente de *C. montrouzieri*, *T. notata* mantêm o padrão de coloração comum das joaninhas, os élitros apresentam um tom marfim a amarelo com manchas circulares de coloração marrom escuro a preto, e apresentam um claro dimorfismo sexual, onde as fêmeas possuem duas manchas pretas em forma de triângulos localizados entre os olhos e são geralmente maiores que os machos. Estes por sua vez apresentam uma mancha branca em forma de triângulo na porção marginal do mesoesterno, e têm a porção final do abdome mais arredondado (Mulsant 1850).

Interação Coccinellidae – Sternorrhyncha

A resposta dos Coccinelídeos a este tipo de presa é semelhante à de outros inimigos naturais, apresentando um padrão de procura composto pela localização inicial de habitats adequados (plantas), busca de presas em plantas e captura e aceitação de presas (Hodek 1993, Souza *et al.* 2019). No entanto, algumas presas capturadas podem não ser adequadas para a manutenção e reprodução das espécies e podem até ser prejudiciais (Hattingh & Samways 1991, Souza & Bezerra 2019). Por exemplo, a joaninha *T. notata* foi detectada sobre colônias da cochonilha-do-carmim, *Dactylopius opuntiae* (Cockerell) (Hemiptera: Dactylopiidae) em plantas de palma forrageira, mas essa presa não permite que as larvas completem o seu desenvolvimento devido ao ácido carmínico (Barbosa *et al.* 2014). Por tanto, a interação predador-presa, pelo

menos nos adultos é mediada pela captação de sinais visuais e químicas associadas com as presas visando uma melhor escolha para aumentar o fitness desses indivíduos. No entanto, tem sido sugerido que a busca por presas em plantas parece ser aleatória, até que o contato com uma presa seja feito (Fisher *et al.* 1999). Atualmente, se conhece que os coccinelídeos podem discriminar entre estímulos coloridos (Mondor & Warren, 2000), orientar-se em relação a voláteis liberados por plantas particulares infestadas com suas presas regulares, discriminar entre plantas com danos mecânicos e plantas infestadas de presas (Yoon *et al.* 2010) e as fases de desenvolvimento das suas presas na planta (Yoneya *et al.* 2009).

No caso das espécies que se alimentam de presas que produzem cera corporal, as interações são mediadas além da percepção de estímulos químicos, pelo desenvolvimento de estruturas como as ceras, especialmente nas fases de maior vulnerabilidade do predador (Larva e pupa) (Pope 1979, Hodek 1993). A cera que cobre o corpo das larvas e as pupas são usadas como estratégias de defesa contra ação de outros predadores como as formigas (Völkl & Vohland 1996, Pérez-Rodríguez & Messelink 2023) e auxiliam no processo de forrageamento evitando elicitar uma resposta negativa na presa (Agarwala & Yasuda 2001).

Dada a associação que apresentam os Sternorrhyncha com as formigas, devido ao recurso açucarado que eles oferecem "honeydew" (Majerus *et al.* 2007, Hodek & Honek 2009), os coccinelídeos apresentam interações competitivas com as formigas, as quais apresentam agressão tanto para adultos como para larvas. No caso dos adultos, estes são afugentados das colônias das presas, enquanto as larvas podem ser feridas, removidas da colônia ou mortas (Jiggins *et al.* 1993, Sloggett & Majerus 2003). Portanto a produção de cera resulta um mecanismo de defesa, que apesar de representar um custo metabólico nos coccinelídeos (Pachecho 2012), favorece a aquisição de alimento. Alguns autores mostraram que a produção de cera nos coccinelídeos,

pode também estar associada a uma camuflagem química ao sequestrar e mimetizar os compostos que produzem suas presas, por sua vez favorece a coexistência facultativa com formigas (Witte *et al.* 1990, Majerus *et al.* 2007). Aparentemente, esta mirmecofilia nos coccinelídeos favorece o estabelecimento dos mesmos em campo, sendo observado uma maior sobrevivência dos predadores em colônias de presas auxiliadas por formigas, dadas que estas os identificam como parte da colônia e as protegem de outros organismos como predadores e parasitoides (Majerus *et al.* 2007).

Relevância do estudo

O controle biológico, entendido como o uso de organismos vivos para a regulação do equilíbrio populacional de espécies daninhas as atividades humanas, promove entre seus pilares a criação massal e liberação periódica de inimigos naturais, comumente insetos entomófagos, como estratégia de controle de insetos fitófagos (Parra *et al.* 2002). Em condições naturais estes organismos promovem a regulação das populações de insetos-praga a níveis que não geram um dano econômico e facilitam o estabelecimento de um equilíbrio ecossistêmico (Whitcomb 1981, Carvalho *et al.* 2019). No entanto, devido as modificações ambientais e antrópicas do entorno, e a introdução de novas pragas, muitas espécies de inimigos naturais não se encontram em quantidades suficientes para regular as populações de pragas, com isso, liberações periódicas são necessárias para incrementar o nível populações de organismos nativos de forma efetiva e para promover o controle das pragas (Parra *et al.* 2002).

A liberação de espécies predadoras como os Coccinelídeos é uma de estratégia amplamente usada no controle biológico, pelo alto potencial das espécies desta família para manter as populações de diversas pragas sobre controle, como de cochonilhas, pulgões, psilídeos,

mosca-branca e algumas pequenas lagartas, entre outras presas. No Brasil, uma das espécies importantes usadas nos agroecossistemas é *C. montrouzieri*, exótica e produzida em criações massais para o controle de cochonilhas farinhentas. Além dela existem outras espécies como *T. notata*, espécie nativa e importante também na regulação de pragas como os Pseudococcidae. No entanto, esta última é pouco usada em programas aplicados de controle devido à pouca informação referente à sua biologia e ecologia e a falta de tecnologia para o estabelecimento de criações massais.

Para entender e predizer a efetividade no controle biológico baseado na introdução de um inimigo natural, como os coccinelídeos em um novo ambiente, é necessário conhecer aspectos de sua biologia e ecologia (Stiling 1993, Venzon *et al.* 2019). Os fatores que determinam o comportamento predatório desses insetos, como por exemplo: o condicionamento pre-imaginal e a experiência dos mesmos, o valor nutricional das presas e as relações mutualistas que estas apresentam com outros organismos (Caubet *et al.* 1992, Souza *et al.* 2019), normalmente coordenadas por sinais químicas (Semioquimicos) ou físicas (Pettersson 2012), podem interferir na eficiência dos mesmos em um programa de controle biológico. Vários estudos mostram que herbívoros, como as cochonilhas, com a presença de ceras e emissão de voláteis que desencadeiam comportamentos de forrageamento e oviposição nos coccinelídeos. No entanto, é pouco conhecido que fatores realmente modulam a interação predador-presa, e como o comportamento e desenvolvimento do predador pode ser alterado ao se alimentar de outra presa. Atualmente alguns trabalhos sugerem que existe uma relação entre as estruturas de defesa (ceras) produzidas pelos coccinelídeos nas fases larvais e pupal e a interação de outros organismos com as presas que consomem, como parasitoides e formigas. No entanto, pouco se conhece sobre a influência do tipo de presa na produção de cera nos predadores, e qual é o custo desta defesa.

Portanto, o objetivo deste estudo foi avaliar como a interação predador-presa por várias gerações sobre as mesmas condições, pode modelar as respostas comportamentais do predador aos estímulos químicos e físicos de um tipo de presa, e quais são os custos metabólicos associados às estratégias de defesa, como a cera, que surgiram como produto da interação presa-predador oligofago. Para atingir esse objetivo, a tese foi dividida em dois artigos. O primeiro teve como objetivo avaliar o efeito do condicionamente pre-imaginal sobre o comportamento de forrageamento e consumo de presas de *T. notata* e *C. montrouzieri*, enquanto o segundo artigo analisou os custos metabólicos associados à produção da cera nas fases de larva e pupa nos coccinelídeos, e como as presas nas quais foram criados influenciaram a composição química dessa cera.

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Material Suplementar

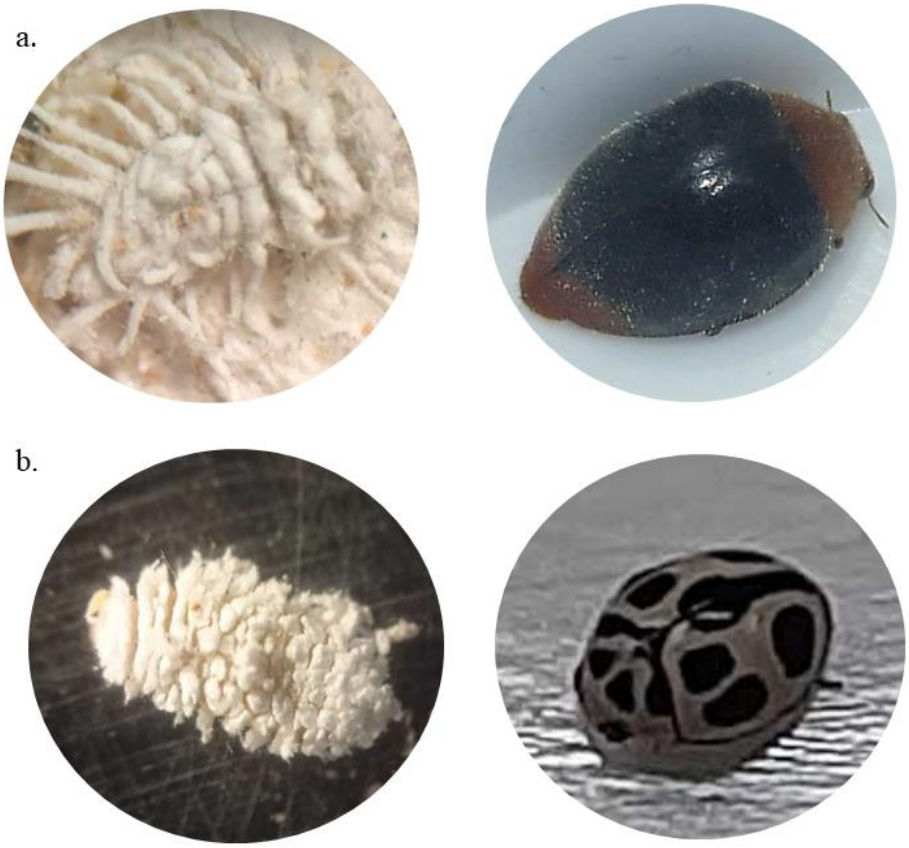

Figura 1. Joaninhas coccidófagas. a. Larva de IV instar e adulto de *Cryptolaemus montrouzieri*. b. Larva de IV instar e adulto de *Tenuisvalvae notata.*

Figura 2. Colônia de *Tenuisvalvae notata*, mostrando como a produção de cera das larvas das joaninhas coccidófagas facilitam a camuflagem com sua presa *Ferrisia dasylirii*.

CAPÍTULO 2

BEHAVIORAL RESPONSES OF LADY BEETLES, *Cryptolaemus montrouzieri* AND *Tenuisvalvae notata* (COLEOPTERA: COCCINELLIDAE) TO SPECIFIC MEALYBUG PREY

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ABSTRACT – Lady beetles are important biological control agents that are considered generalist predators, although some tribes, such as in Scymnini, are specialized in predation of sucking pests, such as mealybugs, aphids and whiteflies. However, prey preference or pre-imaginal conditioning can occur as coccinellids are subjected to large-scale rearing. Thus, predator-prey interaction can be an outcome of conditioning to prey stimuli. To evaluate possible pre-imaginal conditioning, population lines were created for the lady beetles, *Cryptolaemus montrouzieri*, and *Tenuisvalvae notata* where each was fed either mealybugs *Ferrisia dasylirii* or *Planococcus citri* for at least eight generations. Next, the behavioral response of these coccinellids was measured in arenas treated with prey volatiles or footprints, regarding: walking time, walking speed, walking distance, and residence time. Finally, food preference between prey species was measured in the laboratory and semi-field conditions on infested cotton plants. Coccinellids responded similarly to volatiles released by either prey species offered. Furthermore, there were no changes in predator behavior towards either prey footprints. Finally, both predator species consumed more *P. citri* in all tests, regardless of rearing prey indicating no pre-imaginal conditioning. These results suggest that rearing prey not affect the predator behavior, and both coccinellid species are efficient in the biological control of mealybugs.

KEY WORDS: Coccidophagous predator, food preference, pre-imaginal condition, prey recognition

RESPOSTA COMPORTAMENTAL DAS JOANINHAS *Cryptolaemus montrouzieri* E *Tenuisvalvae notata* A COCHONILHAS ESPECÍFICAS

RESUMO – As joaninhas são importantes agentes de controle biológico considerados predadores generalistas, apesar de que algumas tribos como em Scymnini são especializados na predação de pragas sugadoras como as cochonilhas, pulgões e moscas-brancas. Contudo, a preferência de presa ou condicionamento pré-imaginal pode ocorrer nas joaninhas produzidas em larga escala. Então, a interação presa-predador pode ser um resultado do condicionamento a estímulos da presa. Para avaliar um possível condicionamento pré-imaginal, foram estabelecidas populações das joaninhas *Cryptolaemus montrouzieri* e *Tenuisvalvae notata*, cada uma alimentada com as cochonilhas *Ferrisia dasylirii* ou *Planococcus citri*, por no mínimo oito gerações. Em seguida, a resposta comportamental dos coccinelídeos foi avaliada em arenas tratadas com voláteis ou rastros das presas, em relação ao tempo de caminhada, velocidade, distância percorrida, e tempo de residência. A preferência alimentar entre as espécies de presas foi avaliada em condições de laboratório, e semi-campo em plantas de algodão infestadas. Os coccinelídeos responderam similarmente aos voláteis liberados pelas presas ofertadas. Além disso, não houve mudança no comportamento do predador aos rastros das presas. Por fim, ambas espécies de predador consumiram mais *P. citri* em todos os testes, independente da presa usada na criação, indicando ausência de condicionamento pré-imaginal. Esses resultados sugerem que a presa usada na criação pode não afetar o comportamento do predador, e ambas as espécies de coccinelídeos são eficientes controladores biológicos de cochonilhas farinhentas.

PALAVRAS-CHAVE: Predador coccidófago, preferência alimentar, condicionamento pré-imaginal, reconhecimento da presa

Introduction

Adaptive plasticity is a process that includes changes in species genotype in response to interactions with other species and environmental factors over long periods of time (Jermy, 1984; Bernays, 1991; Miner et al., 2005; Futuyma, 2009). These adaptations can modify phenotypic traits, like behavioral, morphological, and physiological aspects that are easily noticed with species that have antagonistic interactions, such as herbivory, predation, or parasitism (Freeland & Boulton, 1992; Futuyma, 2009). Some of the morphological modifications found in insects are the development of mechanoreceptors, chemoreceptors, and changes in mouthpart morphology due to the needs of host/prey finding and selection (Bernays, 1991). Moreover, the aspects linked to species interaction have been chiefly studied between plants and herbivore insects regarding changes in plant structure and chemistry in response to insect damage (Jermy, 1984; Bernays, 1991). However, these findings do not mean that some phenotypic adaptation cannot happen at higher trophic levels with more generalist feeders, such as insect predators, which respond to prey quality and defense as well (Rana et al., 2002; Omkar & James, 2004). Predator-prey interactions lead to adaptations in the behavior of species involved to optimize foraging and increase the energy efficiency of other vital processes (Hassell & May, 1986; Futuyma & Moreno, 1988; Stiling 1988; Schenk & Bacher, 2002). Nevertheless, in most cases, studies on higher trophic levels are restricted to insect parasitoids, due to their specificity with their host (Vinson, 1976; Morawo & Fadamiro, 2019). It may be due to their oligophagous feeding habit and the necessity to feed on many prey to complete development (Hassell & May, 1986).

The predators use chemical cues, like volatiles or prey pheromones to find their prey, but they specially depends on visual cues, such as shape, size, and color to find their prey (Kemp & Cottrell, 2015). Nonetheless, some studies have found that the interaction between the

environment where the predator develops and the prey they fed on could cause an effect on their behavior, suggesting a pre-imaginal conditioning related to the type of prey they fed during development (Jayanthi et al., 2010; Finlay-Doney & Walter, 2012; Urbina et al., 2018).

Pre-imaginal conditioning is commonly found in nature and could be considered a previous event to plasticity adaptation as a consequence of species evolution (Bologna & Di Giulio, 2011; Ramírez et al., 2016). Therefore, for insect populations kept under stable environmental conditions (i.e., laboratory) feeding on the same prey/host continuously, it is expected that they may show variations in biological traits and food preferences. For instance, *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae) showed changes in its attack efficiency when reared for more than 30 generations on an alternative prey (Boycheva et al., 2019), or *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae) showed that only larvae that ate the same prey for years, adopted intensive searching movements after feeding (Ettifouri & Ferran, 1993).

In augmentative biological control, the mass release of natural enemies in a target area is the most used strategy for managing insect pests (Chambers, 1977). The success of this strategy depends on various factors such as the ability of a natural enemy to find its prey/host in the release site and its feeding preference for the target pest (Huffaker et al., 1971; Hokkanen & Sailer, 1985). In this context, lady beetles are largely used in the biological control of crop pests due to their large adaptation capacity and the number of prey species they consume (Hodek & Honek, 2009). For instance, the lady beetles *Tenuisvalvae notata* (Mulsant) and *Cryptolaeumus montrouzieri* Mulsant (Coleoptera: Coccinellidae) have been used in biological control programs for mealybugs (Hemiptera: Pseudococcidae) in different areas of the world (Neuenschwander, 2001; Kairo et al., 2013). However, the constant rearing of those predators in insectaries on

alternative single prey may cause pre-imaginal conditioning and affect their predation efficiency on target prey after release. Therefore, this study evaluated the possible prey preference of *T. notata* and *C. montrouzeri* and behavior mediated by semiochemicals cues on two mealybugs pests, *Planococcus citri* and *Ferrisia dasylirii* (Hemiptera: Pseudococcidae), under laboratory and semi-field conditions.

Material and Methods

Plants. Cotton plants cv. IMA 2106GL, were cultivated in plastic pots of 10 L volume filled with a mixture of soil and humus (2:1), and 7 g of N:P:K fertilizer (20-10-20 Fertine®). Three seeds were planted per pot, 1 cm deep, thinning to two plants if all three germinated. Plants were kept in a greenhouse and irrigated daily until 5-6 fully expanded leaves that were further used in the bioassays.

Insects.

Mealybugs. *Ferrisia dasylirii* (Cockrell) and *Planococcus citri* Risso (Hemiptera: Pseudococcidae) colonies were reared on pumpkins var. "Jacarezinho" according to Sanches & Carvalho (2010). Thus, pumpkins were rinsed with soap, sodium hypochlorite 1%, and water, dried, and placed in plastic trays lined with a paper towel. Next, the pumpkins were infested in the peduncle with gravid mealybug females from the colony. Each species was kept separate into different acrylic cages. Emerging nymphs colonized the pumpkin surface until its complete infestation. At this point, new non-infested pumpkins were placed on the top of the infested ones to allow the infestation by mealybugs and keep the colony going. Each population was kept separately under a temperature of 25 ± 2 °C, relative humidity of 60 ± 10%, and a photoperiod of 12:12 hours (L:D). Mealybugs *F. dasylirii* were collected from cotton plants (Recife,

Pernambuco, Brazil 8.017070°S, 34.944362°W), whereas *P. citri* was collected from commercial *Anona* spp. (Annonaceae) orchards located in several production areas of Pernambuco state (Agreste, Zona da Mata, São Francisco and Sertão), North-East Brazil, and identified by Vitor Cezar Pacheco da Silva (Da Silva et al., 2019).

Lady beetles. Adult *T. notata* were collected from cotton plants infested with the cotton mealybug *Phenaccocus solenopsis* Tinsley (Surubim, Pernambuco, Brazil, 7.833056°S, 35.754722°W) and identified by José Adriano Giorgi (Barbosa et al., 2014a). As for *C. montrouzieri*, adults originated from a colony kept in EMBRAPA Semiárido (Empresa Brasileira de Pesquisa Agropecuária), Petrolina, Pernambuco state, Brazil. The colonies of the lady beetles were kept separately inside transparent PlexiglassTM cages (40 × 25 × 20 cm), containing lateral openings closed by 'voil' fabric for internal ventilation. The cage was lined with a paper towel and received a mealybug-infested pumpkin as prey. Each lady beetle species (*T. notata* and *C. montrouzieri*) was reared separately on each prey species (*F. dasylirii* and *P. citri*). Therefore, there were four colonies of lady beetles, according to the respective predator-prey combination, and reared under these conditions for at least eight generations previously to the bioassays (240 days approx.). Virgin lady beetle adults, 5-10 days-old, were used in the following tests, the adults used for the test were previously isolated in Petri dishes to be sure of the lady beetle age for the experiment.

Response of lady beetles to prey volatiles. This test was carried out to determine whether the lady beetles can detect the presence of prey by volatile cues. Preliminary tests on the laboratory have shown that the lady beetles were not responsive in an olfactometer with an airflow (unpublished data). Therefore, a three-way closed arena was used to test the ability of lady beetles to detect volatiles by diffusion. The test arena comprises a Plexiglass^{TM} base (20 x 20 x

1.2 cm) and a glass top (bottom of a Petri dish of 15-cm diameter, Pyrex). The base contained three rectangular wells (1 x 2 x 1 cm), positioned at 8 cm distance from each other as an equilateral triangle (Silva-Torres et al. 2005). There are three circular marks (4-cm diameter) on the glass top right above the wells. Each well received randomly one treatment, according to the following combinations offered simultaneously: i) blank (empty), glass rod ("dummy"), and *P. citri* (150 nymphs and adults); ii) blank, glass rod, and *F. dasylirii* (150 nymphs and adults); iii) blank, *F. dasylirii* (150 nymphs and adults), and *P. citri* (150 nymphs and adults). A circular filter paper (20-cm diameter) (CELAB®) was placed over the arena base to cover the wells and remove visual and physical cues, whereas it allowed for the diffusion of volatiles from the treatments below. Thus, a single lady beetle was placed in the center of the arena on the filter paper, and the glass top closed the system. The lady beetle was allowed to acclimate inside the arena for one minute, and its behavior was observed for the next ten minutes. The elapsed time to detect each area above the wells and the time spent on each area was measured. In this experiment 30 replicates were used for each lady beetle species (*T. notata* and *C. montrouzieri*), sex (male and female), and prey (*F. dasylirii* and *P. citri*); therefore, we have n=180 for treatment. The arena was rotated after each trial to avoid possible treatment position bias. Each replicate received a new clean paper. Adult lady beetles were starved for 24 hours before the bioassay to stimulate the foraging behavior , exposed to the prey volatiles in the arena, and tested only once.

Time spent by lady beetles around the respective treatment patches in the arena was analyzed through the Kruskall-Wallis test using PROC NPAR1WAY of SAS (SAS Institute, 2002). The effect of sex in the response of lady beetles was subjected to two-way ANOVA considering treatments and sex of individuals using the Proc GLM of SAS (SAS Institute, 2002).

Data for the first choice made by lady beetles and the number of responding adults to a treatment or controls were analyzed using the non-parametric Proc FREQ (SAS Institute, 2002) followed by χ^2 tests ($\alpha = 0.05$).

Response of lady beetles to prey footprints. These tests aimed to evaluate the capacity of the lady beetles to recognize the trails left by prey on filter paper (CELAB®). Footprints were obtained by allowing female mealybugs to walk freely on the filter paper for 24 hours, which were used in the bioassays immediately after, as described next. Thus, Petri dishes (9-cm diameter) were used as test arenas according to Spíndola et al. (2013), and the bottom of the arena was lined with filter paper. Paired treatments were offered simultaneously to the lady beetles in the arena, according to the areas of the filter paper [area 1 (A1): area 2 (A2)] as follows: ***Test 1)*** Partially treated arena= i) footprints of *P. citri* x untreated area (Control area - no footprints); ii) footprints of *F. dasylirii* x untreated area; ***Test 2)*** footprints of *P. citri* x footprints of *F. dasylirii*, and ***Test 3)*** untreated x untreated area (double control). The positions of the areas offered in the arenas were rotated after every five replicates, to avoid any interference of position in the behavior of the lady beetles. Also, each repetition was made in a new Petri dish, to reduce the risk of contamination by other prey cues. Similarly, to the previous test, there were 30 replicates for each lady beetle species (*T. notata* and *C. montrouzieri*), sex (male and female), and prey (*F. dasylirii* and *P. citri*). Adult lady beetles were starved for 24 hours before the bioassay to equalize hunger level. The lady beetle was released in the center of the arena, on the filter paper, and allowed 5 minutes of acclimation. Then, its behavior was observed for 10 minutes with the software ViewPoint™ (ViewPoint Life Sciences Inc., Montreal, Canada). The behavioral parameters observed were walking time, walking distance, walking speed, and the

number of stops on each half (A1 and A2) of the filter paper. Each lady beetle was used only once.

For each predator-prey combination and test, the variables of walking time, walking distance, walking speed, and the number of stops were analyzed by Kruskall-Wallis test using PROC NPAR1WAY of SAS (SAS Institute, 2002). To determine the occurrence of arrestment (permanence time) of lady beetles in partially treated arenas with footprints of each prey were performed the non-parametric Proc FREQ (SAS Institute, 2002) was followed by χ^2 tests (α = 0.05). To evaluate the ladybird sex effect in the prey footprint response, the data of each combination were analyzed through the Kruskall-Wallis test using PROC NPAR1WAY of SAS (SAS Institute, 2002). Meanwhile, the variables across the four combinations were analyzed by MANOVA using PROC GLM of SAS (SAS Institute, 2002). Means were separated by the Tukey HSD test ($\alpha = 0.05$), where four means were compared. In all tests, α = 0.05 of probability.

Lady Beetle prey preference – This test was designed to evaluate whether there is pre-imaginal conditioning in food preference by *T. notata* and *C. montrouzieri*.

Lab test - The type of prey preferred by IV instar larvae and adults of lady beetles was determined in the laboratory according to Ferreira (2019). As previously stated, lady beetles originated from the different predator-prey colony combinations maintained in the laboratory. Thus, IV instar larvae and virgin adults (5-days old) were starved for 24 hours before choice tests to equalize hunger and stimulate feeding. Next, for free choice tests, lady beetles were singly placed in Petri dishes (9-cm diameter) lined with a filter paper (CELAB®) and offered

simultaneously 10 third instar nymphs of the mealybugs, being five of *F. dasylirii*, and five of *P. citri,* respectively. For non-choice tests, the lady beetles received 10 nymphs of only one prey species, either *F. dasylirii* or *P. citri*. There were 50 replicates for each predator-prey treatment combination (50 larvae, 50 adults (25 males and 25 females). After 24 hours, the number of prey consumed per mealybug species was recorded by the difference between the number of prey offered and left in the arena.

The data were tested under the null hypothesis that no pre-imaginal conditioning implies an equal number of mealybug nymphs preyed (1:1 ratio), regardless of what was the prey on which the predator had been reared on. The analyses of the free-choice test were performed using the non-parametric Proc FREQ (SAS Institute, 2002) followed by χ^2 tests ($\alpha = 0.05$). In the *No-choice* test to determine the differences in the prey consumption between sex (male, female, and larvae) predator-prey combination, the data were analyzed through the Kruskall-Wallis test using PROC NPAR1WAY of SAS (SAS Institute, 2002). The prey-consumption response of lady beetles was subjected to two-way ANOVA, considering the treatments (prey reared) and sex of individuals (male, female, and larvae) as factors, using the Proc GLM of SAS. Variables levels means were separated by the Tukey HSD test ($\alpha = 0.05$) (SAS Institute, 2002). In all tests, α = 0.05 of probability.

Semi-field test – In this test, we evaluated the predation rate of the lady beetles in a greenhouse in free-choice and non-choice tests after 24 and 48 hours. Thus, we used cotton plants (30 days-old) infested with nymphs of *F. dasylirii* and *P. citri*. To do so, the upper leaf of the plant was used as an infestation site, which occurred 24 hours before the test. In the *free-choice test* a circular filter paper (9-cm diameter) (CELAB®) containing 20 mealybug nymphs, 10 of each species, was attached to the leaf with a metal clip to the upper expanded leaf, being one paper disc per leaf,

and inserted in a fine-mesh cage (18 cm wide x 24 cm length). The next day, the number of nymphs per cage was checked and when needed, additional individuals were added to assure that 20 nymphs were present per each leaf cage. Virgin adult lady beetles, males and females in the same proportion, were used in this bioassay. They were starved for 24 hours prior to tests to stimulate the feeding behavior. Next, one lady beetle was released inside each cage according to the predator-prey combination. 20 females and 20 males were used per each predator-prey combination, and per prey offered. In total for the test were used to 320 plants for each time separately. The number of prey consumed was measured after 24 and 48 hours. In the *non-choice test*, the procedure was like the free-choice test, but leaves were infested with 20 nymphs same mealybug species per leaf cage, either *F. dasylirii* or *P. citri*, in this case, there were used 40 replicates (20 females and 20 males) per predator-prey combination, totalizing 160 plants for predation time. All treatments and repetitions were made on the same day, to reduce the environmental effect on the data, the variables temperature and relative humidity were 29.61 ±3.17 and 69.83 ± 11.62, respectively. The analyses of *free-choice tests* were performed using the non-parametric Proc FREQ (SAS Institute, 2002) followed by χ^2 tests ($\alpha = 0.05$). In the *non-choice test*, to determine the differences in the prey consumption between sex predator-prey combinations, the data were analyzed through the Kruskall-Wallis test using PROC NPAR1WAY of SAS (SAS Institute, 2002). The prey consumption response of lady beetles was subjected to two-way ANOVA considering the two factors: predator-prey combination and the lady beetle sex, using the Proc GLM of SAS (SAS Institute, 2002). In all tests, $\alpha = 0.05$ of probability.

Results

Response of lady beetles to prey volatiles. Adult *C. montrouzieri* showed an attraction response to volatiles of prey offered in the three-way arena regardless of prey they were reared on (Table 1). In addition, there was no effect of sex in the predator response to prey volatiles in the three-way arena (when reared on *F. dasylirii*: $F_{(1,174)} = 0.00$; $P=0.95$; when reared on *P. citri* $F_{(1,174)} = 2.45$; $P= 0.11$).

When *C. montrouzieri* was reared on *F. dasylirii* and offered volatiles of the same prey versus control (blank and dummy), it spent 1.62 more times on the area above the prey (*F. dasylirii*). In the same way, *C. montrouzieri* responded when offered volatiles of *P. citri* versus the controls. It spent 1.96 more times on the area above prey (*P. citri).* Moreover, when *C. montrouzieri* was reared on *P. citri*, it spent 2.41 and 2.79 more times in areas above *P. citri* and *F. dasylirii*, respectively, versus the control area (Table 1). However, when offered volatiles of the two species, *C. montrouzieri* did not show any preference (Table 1).

Adults of *T. notata* showed a slight attraction response to volatiles of *F. dasylirii* offered in the three-way arena regardless of prey they were reared on (Table 1). In addition, there was no effect of sex in the response of predators (when reared on *F. dasylirii*: $F_{(1,174)} = 0.26$; $P= 0.60$; when reared on *P. citri* $F_{(1,174)} = 0.09$; $P= 0.76$). As C. *montrouzieri*, adult *T. notata* reared on *F. dasylirii* did show differences between the time spent in the area with *F. dasylirii* prey volatiles and did not show response in the area with *P. citri*, but it was observed differences on the population reared on *P. citri* (Table 1). When offered the volatiles of both prey species simultaneously, *T. notata* did not show a preference for either and spent 1.95 more times above the well of the control (Table 1).

Results of the first choice in the three-way arena showed that initially, predators did not prefer volatiles released by the prey. Overall, there was no significant difference in the number of times the lady beetles chose the prey versus the controls (blank and dummy). Except for *C. montrouzieri* reared on *F. dasylirii*, subjected to volatiles of *F. dasylirii* + *P. citri* versus blank control, 70% of the insects went towards any of the prey on the first choice (χ^2 = 5.0; DF =1; P< 0.05). When *T. notata* was reared on *P. citri*, 49% of the insects were toward the controls in comparison to *P. citri* at the first-time choice, whereas the other treatment combinations did not show a preference for any of the areas, with controls or prey (χ^2 = 13.37; DF =1; P< 0.001).

Response of lady beetles to prey footprints. ***Test 1)*** Partially treated arena: i) footprints of *P. citri* x control area (untreated); ii) footprints of *F. dasylirii* x control area.

In most of the cases, *F. dasylirii* did not affect the response of *C. montrouzieri* adults to prey footprints, except for walking distance (WD) ($F_{(1,58)}$ = 10.06, P= 0.001) and walking time (WT) ($F_{(1,58)}$ = 4.36, P< 0.05) towards the untreated area (Supplementary table S1a-d). On the other hand, when *C. montrouzieri* was reared on *P. citri* and offered footprints of *F. dasylirii*, there was a significant difference in all the variables measured, showing a preference toward the area with prey footprint, except for the WD ($F_{(1,58)}$ = 1.84, P= 0.17). Similarly, when it was offered footprints of *P. citri*, in comparison to the untreated area, there was an effect on the WT ($F_{(1,58)}$ = 5.12, P< 0.05) and WD ($F_{(1,58)}$ = 6.62, P= 0.01) (Supplementary table S1a-d).

For *T. notata* reared on *F. dasylirii*, there was no significant difference in most the variables measured (Supplementary table S1a-d), except for WT ($F_{(1,58)}$ = 6.49, P= 0.01) of those offered *P. citri* footprints. Similarly, *T. notata* reared on *P. citri*, when offered footprints of *F. dasylirii* versus untreated areas, there was significant effect in NS ($F_{(1,58)}$ = 8.10, P< 0.05)

on the area treated with the footprints of *P. citri* (Supplementary table S1d), to all other parameters there was no difference.

Test 2) Footprints of *P. citri* x footprints of *F. dasylirii*. Lady beetles *C. montrouzieri* (reared on *F. dasylirii*: $\chi^2 = 2.36$, DF = 1, $P = 0.12$; reared *P. citri*: $\chi^2 = 0.16$, DF=1, $P = 0.68$) and *T. notata* (reared on *F. dasylirii*: $\chi^2 = 1.86$; DF = 1, $P = 0.17$; reared on *P. citri* $\chi^2 = 0.32$; DF = 1, $P = 0.56$), spent a similar amount of time (WT) on areas treated with footprints of either prey (Figure 1 and Figure 2). Also, there was no effect of *C. mountrouzieri* sex on WT, WD, WS and NS, regardless of prey that they were reared on (Supplementary Table S1a-d). Significant effects were found for WT ($F_{(1,58)} = 4.20$, P= 0.04) of *C. montrouzieri* reared on *F. dasylirii*, and NS ($F_{(1,58)} = 16.56$; P< 0.001) of *C. montrouzieri* was reared on *P. citri* (Figure 1).

Overall, results were similar for *T. notata* reared on *F. dasylirii* and subjected to footprints of both mealybug species. There was an effect just on walking speed (WS) ($F_{(1,58)} = 4.11$; P= 0.04). Also, when *T. notata* was reared on *P. citri*, there was no difference in any of the variables measured (Figure 2). Furthermore, the sex of *T. notata* adults did not affect the lady beetle response to any of the variables, regardless of the prey they were reared on (Supplementary Table S1a-d).

Test 3) Untreated area x Untreated area (double control). When the lady beetles were subjected to untreated areas in the arena, as a control for confounding effects such as side position, there was no difference in the variables WT, WD, WS and NS, regardless of the prey predators were reared on (Supplementary Table S1a-d).

Lady beetle prey preference. *Lab test*: For *C. montrouzieri*, there was a significant difference in *F. dasylirii* consumption according to the developmental stages and the prey they were reared on (Table 2). When *P. citri* was offered, there was no significant difference regarding developmental stages ($F_{(2,217)} = 0.32$; $P = 0.73$) (Table 2). In addition, when *C. montrouzieri* larvae were reared on *F. dasylirii* or *P. citri* and they were offered both prey (*P. citri* and *F. dasylirii*), there was no difference in the number of prey consumed (Figure 3). In contrast, *C. montrouzieri* adults (male and female) preferred to feed on *P. citri*, regardless of the prey they were reared on (Figure 3).

For *T. notata*, there was a significant effect in *F. dasylirii* consumption according to their developmental stage and the prey they were reared on (Table 2). Also, consumption of *P. citri* by *T. notata* was affected by the prey species it had been reared, but no differences between the developmental stages ($F_{(2,217)} = 213.65$; $P = 0.11$) (Table 2). In addition, larvae of *T. notata* reared on *F. dasylirii* did not show preference for consumption of either prey ($\chi^2 = 0.45$, DF = 1, $P = 0.50$). On the other hand, larvae of *T. notata* reared on *P. citri* preferred to feed on *P. citri* ($\chi^2 = 4.31$, DF = 1, $P < 0.05$) (Figure 4). Adults *T. notata* (males and females) preferred to feed on *P. citri*, no matter the prey they consumed during development (Figure 4).

***Semi-field test*:** *Free-choice test* – After 24 hours exposure, *C. montrouzieri* females reared on *P. citri* that preferred to prey on *P. citri* ($\chi^2 = 3.84$, DF =1, $P < 0.05$), for all other treatments adults (male and female) did not show preference between prey offered (*F. dasylirii* and *P. citri*), regardless of the prey they were reared on, (Figure 5A). In contrast, *T. notata* adults (male and female) reared on *P. citri* did not show preference for either prey, whereas *T. notata* adults (male and female) reared on *F. dasylirii* preferred *F. dasylirii* (Figure 5A). After 48 hours

of exposure, overall, there was no significant difference in prey consumption (*F. dasylirii* and *P. citri*), regardless of prey they were reared on, or the prey offered (Figure 5B).

No-choice test - After 24 hours exposure, when *C. montrouzieri* adults were offered a single prey, the predation response was different by the sex and prey they were reared on (Table 3). Moreover, *C. montrouzieri* males reared on *F. dasylirii* preyed on 10.5% more nymphs of *F. dasylirii* than males reared on *P. citri* (χ^2 = 29.22, DF =1, P< 0.0001), and the females preyed an equal number of that nymph, no matter the prey they were reared on (χ2 = 0.10, DF =1, P= 0.75). Also, *C. montrouzieri* males reared on *F. dasylirii* preyed 37.4% more on nymphs of *P. citri* than males reared on *P. citri* (χ^2 = 28.49, DF =1, P< 0.001). Similarly, females reared on *F. dasylirii* preyed 10.5% more on nymphs of *P. citri* than females reared on *P. citri* (χ2 = 7.94, DF =1, P< 0.004) (Table 3).

For *T. notata* after 24 hours, in general, the consumption of nymphs of *F. dasylirii* was not affected by the prey they were reared on and for the sex of the lady beetle (Table 3). In contrast, when the prey was *P. citri*, the consumption was affected just by the sex of the lady beetle (Table 3). Thus, *T. notata* males reared on either prey did not show difference in prey consumption upon *P. citri* (χ^2 = 0.61, DF =1, P= 0.43). However, *T. notata* females reared on *P. citri* preyed 17.44% more than prey than females reared on *F. dasylirii* (χ^2 = 10.67, DF =1, P= 0.001).

After 48 hours exposure of *C. montrouzieri* in the non-free choice test, when *P. citri* was offered the predation response was different between male and female. When the prey offered was *F. dasylirii*, there was a significant effect only by the predator sex (Table 3). In contrast, *C. montrouzieri* adults reared on *F. dasylirii* preyed on more nymphs of *P. citri* than those reared on

P. citri (35.5% more on males: $\chi^2 = 29.20$, DF =1, $P < 0.001$; 8.3% more for females: $\chi^2 = 6.53$, DF =1, $P < 0.05$).

For *T. notata*, the consumption after 48 hours was affected by the predator sex and the prey that they were reared on, when the prey was *F. dasylirii*,, but when the prey offered was *P. citri*, there was a significant effect only for the predator sex (Table 3). Specifically, for *T. notata* males, after 48 hours exposure in the non-free choice test, they consumed a similar number of nymphs regardless of the prey they were reared on (*F. dasylirii* prey: $\chi^2 = 3.74$; DF =1, $P = 0.05$; *P. citri* prey: $\chi^2 = 0.47$, DF =1, $P = 0.49$) (Table 3). On the other hand, *T. notata* females reared on *F. dasylirii* showed 19.5% and 17.7% more consumption of *F. dasylirii* and *P. citri* nymphs, respectively, than those reared on *P. citri* (*F. dasylirii* prey: $\chi^2 = 8.37$; DF =1, $P < 0.05$; *P. citri* prey: $\chi^2 = 13.98$, DF =1, $P < 0.001$) (Table 3).

Discussion

The constant and large-scale insect colony-rearing in insectaries, such as production of biological control agents, can change the predation efficacy of natural enemies as a by-product of pre-imaginal conditioning on a factitious/alternative prey/host (Boller, 1972; El-Wakeil, 2007). On the other hand, our study shows that for generalists or oligophagous predator species, such as the lady beetles examined in this study, the pre-imaginal conditioning does not happen, and the prey used on rearing does not affect the predation behavior or their effectiveness. The lady beetle species used as biological models, *C. montrouzieri* and *T. notata*, did not show major changes in their behavioral responses associated to prey semiochemicals (volatiles and non-

volatiles/footprints), as well as did not show preference to prey on the prey species they had been reared previously and continuously for eight generations.

In the three-way arena where the predators were subjected to in-situ volatiles released by the prey, without visual cues, the lady beetles *C. montrouzieri* and *T. notata* responded similarly to either *F. dasylirii* and *P. citri*. Overall, predators offered both prey species did not show a preference for either prey. The lady beetles *C. montrouzieri* and *T. notata* are predators of coccids and pseudococcids (Kundoo & Khan, 2017; Rondoni et al., 2021). Thus, they respond indistinctly to the cues of their prey. Specialist predators need adaptations to respond to their prey population fluctuations, resulting in a non-advantageous trade-off for the kind of prey they consume (Hassel & may, 1986). Mealybug species are seasonal and sensitive to environmental variables, particularly rainfall, which helps mealybug dispersion and limits their presence in the field (Silva-Torres et al., 2019). Therefore, prey seasonality may limit the permanence of predators that need to consume several prey individuals to complete development.

Interestingly, even though both lady beetle species had similar overall responses, *C. montrouzieri* remained 1.24 times longer than *T. notata* in area with prey cues, and this could be because *C. montrouzieri* adults usually search and stay longer in areas with mealybugs (Villegas-Mendoza et al., 2012) and respond to prey volatiles and change their searching behavior accordingly (Urbina et al., 2018).

However, *C. montrouzieri* showed no preference response to a specific prey. This corroborates results of previous studies, which showed no preferences in the *C. montrouzieri* response, subjected to volatiles of different prey species such as *Pseudococcus araucariarum* (Williams 1985), *Pseudococcus cunninghamii* (Williams 1985) and *P. citri* (Finlay-Doney & Walter, 2012). Similarly, *T. notata* did not prefer either prey. It responded

more towards the control areas, suggesting that this species either has no preference between the chemical signals released by the mealybugs offered here or did not detect those chemical cues.

Insect predators need to find detectable and reliable signals released by their prey to succeed in predation. As the lady beetles did not show preference between prey offered in this study, results also suggest that besides the lack of pre-imaginal conditioning, the chemical profiles of semiochemicals (volatiles and non-volatiles) produced by *F. dasylirii* and *P. citri* may be similar. This could be justified by the fact that these prey species are both Pseudococcidae, reared under the same conditions on pumpkins, and may share similar chemical profiles, and studies have shown that the prey's host can affect the chemical profile of the insects (Liang & Silverman, 2000; Vaníčková et al., 2012). In addition, the lady beetles are generalists, therefore theinteraction with their prey less tight/restrictive than specialist (Strand & Obrycki, 1996). Furthermore, in the prey searching process, the insects evaluate the cues that might indicate the presence of the prey, and consider the energetic cost to respond to these cues (Michel & Adams, 2009; Siepielski et al., 2016), Then the feeding behavior of predators regarding functional response to prey-density, type and developmental stage of prey (Obata, 1997; Sarmento et al., 2007; Barbosa et al., 2014a, b; Ferreira et al., 2020); for *C. montrouzieri*, its searching behavior is associated to a generalized prey search in the whole area, whereas for *T. notata* is characterized by an arrestment response, evaluating other possible cues (e.g. tactile) not accounted for in areas with no olfactory stimuli, or resting behavior.

The lady beetle species examined in this study showed similar behavior when subjected to prey footprints (non-volatile cues), without preference for either prey chemical cues. There was no difference in the predator response with the control areas either, suggesting that the semiochemicals in prey footprints were not enough to elicit foraging behavior by the coccinellids

in the test arenas used here. Similarly, other studies have shown that *C. montouzieri* lacks a response to only one type of prey stimulus, such as volatile cues, when they are limited to walking (as in our tests) instead of flying (Finlay-Doney & Walter, 2012). More complex stimuli, such as the wax produced by mealybugs, offer tactile and olfactory chemical cues for predators (Hashimoto & Kitaoka, 1982; Arunkumar et al., 2018; Ahmad et al., 2020). They work as an elicitor of oviposition and predation (Van den Meiracker et al., 1990; Merlin et al., 1996; Kairo et al., 2013) for lady beetles.

Despite no preference between prey species, predators showed behavioral differences depending on the prey they fed. For instance, *C. montrouzieri* dispersed more/faster and had less number of stops when reared on *F. dasylirii* than those reared on *P. citri*, which had a higher latency to start dispersal. These results indicate *F. dasylirii* may be causing an adaptation or changing the fitness of *C. montrouzieri*, which was reflected in its walking behavior. Rana et al. (2002) reported this change in the lady beetle *Adalia bipunctata* (Linnaeus 1758) when fed on two species of aphids with different nutritional traits. In contrast, for *T. notata*, its walking behavior was not affected by the prey it had been reared on. This suggests that even if there is any nutritional difference in prey species offered, this difference was not enough to cause behavioral changes in *T. notata*, and it seems that both mealybug species do not cause significant changes in the fitness of this lady beetle. This hypothesis is supported by results found by Ferreira et al. (2020), which compared the development and reproduction of *T. notata* upon these mealybug species and found no significant prey effects.

Regarding predation in the laboratory, lady beetle larvae show higher prey consumption than adults (males and females). For *C. montrouzieri*, larvae showed higher predation upon nymphs of prey they fed until the test. On the other hand, this was not the case for *T.*

notata. Furthermore, the older larvae also need to consume more prey than younger ones to meet developmental, nutritional requirements and store energy to enter the pupal stage (Işıkber & Copland, 2001; Finlayson et al., 2010; Kumar et al., 2014). However, lady beetle larvae showed no preference between prey species. Larvae fed a similar number of mealybug nymphs, regardless of the prey they had been reared on. In the case of the predation response for lady-beetle adults, they consumed and preferred to prey on *P. citri* regardless of the mealybug species they were reared. It could be an effect of prey size, as *P. citri* is smaller than *F. dasylirii*, being easier to attack and handle, and the predators need to consume more prey to meet their nutritional and energetic needs (Roger et al., 2000; Chaudhary et al., 2016). Also, *P. citri* might have other traits favoring predation, such as the release of a higher amount of honeydew that can be used as supplemental food (Wäckers et al., 2008; Lundgren, 2009) and tactile/chemical cue to find the prey (Van den Meiracker et al., 1990; Sardoy et al., 2007). Moreover, adult insects seem more adapted to perceive prey cues than larvae (Souto et al., 2021).

The results of the semi-field choice test were consistent with those from laboratory tests. There was no effect of pre-imaginal conditioning on predation upon *F. dasylirii* and *P. citri* at 24- and 48-hours prey exposition. Lady beetles used in this study are coccidophagous with preference, voracity, and high predation upon Coccidae and Pseudococcidae mealybugs (Dixon & Dixon, 2000; Kumar et al., 2019). In addition, females of both lady beetle species showed higher mealybug consumption than males, which can be associated with a nutritional need for reproduction (egg maturation) and dispersal to find a suitable oviposition site, etc (Houck, 1991; Salim et al., 2015). Moreover, in the *No-choice* test, lady beetles consumed more nymphs of *P. citri* on the 24- and 48-hour tests, no matter the prey they were reared on (*F. dasylirii* or *P. citri*). It suggests that to meet the nutritional needs, a greater consumption of this prey is necessary

(Rana et al., 2002; Ferran & Dixon, 2013). Furthermore, the apparent prey preference in the laboratory was not present in the semi-field free-choice test of 48 hours. Thus, this supposed prey preference is not constant or related to pre-imaginal conditioning. However, it could be an artifact of prey availability (Rana et al., 2002; Ferran & Dixon, 2013).

In conclusion, predator response to the group of prey cues (volatile and tactile – footprint) was not constant and was not oriented to a specific prey (*P. citri* or *F. dasylirii*) after being reared on the same prey after eight generations in the laboratory. Therefore, there was no pre-imaginal conditioning of *C. montrouzieri* and *T. notata* upon those prey species, which did not affect their searching, walking, and predatory behavior in laboratory and semi-field tests. Thus, the results indicate that both predator species are potential biological control agents for the species of mealybugs here tested as well as other Coccidae and Pseudococcidae species.

Finally, it is important to consider further investigation of ecological aspects of prey-predator interaction in the field. For instance: Would predator search and predatory behavior change after mass rearing in the laboratory in an alternative prey? Are there any differences in the response of the lady beetle species to prey stimuli, not only individually but as a population in a complex environment where other prey and predator are present? Answers to those questions will shed light on the aspects and possible success of biological control using lady beetles.

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Table 1: Mean time (± SE) spent by adults *Cryptolaemus montrouzieri* (*Cm*) and *Tenuisvalvae notata* (*Tn*) reared on *Ferrisia dasylirii* or *Planococcus citri,* during a 10-min trial in a three-way choice test, responding to various combinations of emitted volatiles of mealybug prey versus controls (dummy and blank).

Predator-prey reared on	**Treatment offered**	**Mean time ± SE (seconds)***	**No. Responding**	**Statistics[a]**
Cm – F. dasylirii	*F. dasylirii*	46.79 ± 8.21a	49	$F_{(2,174)}$= 7.49; *P*< 0.001
	"dummy" control	24.78 ± 5.36b	37	
	Blank control	31.40 ± 5.92b	39	
	P. citri	48.30 ± 11.08a	47	$F_{(2,174)}$= 27.71; *P*< 0.001
	"dummy" control	35.03 ± 6.68b	47	
	Blank control	11.3 ± 3.09b	50	
	F. dasylirii	35.04 ± 7.47a	37	$F_{(2,174)}$= 1.62; *P*= 0.201
	P. citri	39.63 ± 8.11a	44	
	Blank control	28.16 ± 7.49a	32	
Cm - P. citri	*F. dasylirii*	64.14 ± 11.13a	51	$F_{(2,174)}$= 39.21; *P*< 0.001
	"dummy" control	32.24 ± 7.75b	42	
	Blank control	13.67 ±3.22b	35	
	P. citri	68.76 ± 31.48a	41	$F_{(2,174)}$ = 20.65; *P*< 0.001
	"dummy" control	21.23 ± 4.92b	34	
	Blank control	35.89 ± 6.59b	5	
	F. dasylirii	35.62 ± 5.24a	38	$F_{(2,174)}$= 0.27; *P*= 0.76
	P. citri	31.29 ± 6.98a	45	
	Blank control	35.58 ± 5.70a	46	
Tn - F. dasylirii	*F. dasylirii*	64.14 ± 11.13a	4	$F_{(2,174)}$= 3.76; *P*< 0.05
	"dummy" control	27.44 ± 5.75b	39	
	Blank control	30.16 ±7.36b	32	
	P. citri	38.70 ± 9.56a	39	$F_{(2,174)}$= 1.73; *P*= 0.18
	"dummy" control	40.67 ± 8.31a	37	
	Blank control	23.38 ± 7.12a	37	
	F. dasylirii	25.94 ± 6.85a	31	$F_{(2,174)}$= 9.47; *P*< 0.001
	P. citri	21.81 ± 6.11a	31	
	Blank control	46.73 ± 8.78b	40	
Tn - P. citri	*F. dasylirii*	67.25 ± 15.19a	45	$F_{(2,174)}$= 21.82; *P*< 0.001
	"dummy" control	27.82 ± 7.23b	32	
	Blank control	17.84 ± 5.70b	22	
	P. citri	32.98 ± 11.65a	24	$F_{(2,174)}$= 0.04; *P*= 0.958
	"dummy" control	33.95 ± 7.26a	40	
	Blank control	35.29 ± 8.62a	32	
	F. dasylirii	19.94 ±5.99a	27	$F_{(2,174)}$= 8.13; *P*< 0.001
	P. citri	24.55± 6.94a	29	
	Blank control	42.89 ± 7.25b	41	

[a]Means followed by the same lower-case letter in the column within a test prey combination are not significantly different by the Kruskall-Wallis test ($\alpha = 0.05$).

*The mean time was merged between the sexes because there are no significant differences between males and females ($P>0.05$).

Table 2: Average (± SE) number of mealybugs consumed by *Cryptolaemus montrouzieri* (*Cm*) and *Tenuisvalvae notata* (*Tn*) reared on *Ferrisia dasylirii* or *Planococcus citri*, during a 24-hour trial in the laboratory test.

Predator-prey reared on	**Prey offered**	**Stage development predator**			**Statistics [a]**
		Female	Male	Larvae	
Cm – F. dasylirii	*F. dasylirii*	2.2±0.20 Ab*	1.53±0.16 Bc	5.04±0.23 Aa	$F_{(1,216)} = 228.5$; $P < 0.001$
Cm - P. citri		2.6±0.24 Ab	2.26±0.24 Ab	3.84±0.25 Ba	
Statistics[b]		$F_{(2,217)} = 249.76$; $P < 0.001$			
Cm – F. dasylirii	*P. citri*	4.3±0.32 Aa	3.06±0.17 Ba	3.12±0.23 Ba	$F_{(1,216)} = 73.13$; $P < 0.001$
Cm - P. citri		4.6±0.33 Aa	5.56±0.38 Aa	5.98±0.28 Aa	
Statistics[b]		$F_{(2,217)} = 0.32$; $P = 0.73$			
Tn – F. dasylirii	*F. dasylirii*	1.36±0.14Bb	1.66±0.16 Ab	2.66±0.19 Ba	$F_{(1,214)} = 197.56$; $P < 0.05$
Tn - P. citri		2.03±0.26Ab	1.28±0.19 Bc	2.94±0.15 Aa	
Statistics[b]		$F_{(2,217)} = 201.58$; $P < 0.001$			
Tn – F. dasylirii	*P. citri*	2.46±0.18 Ba	2.26±0.16 Ba	2.42±0.15 Ba	$F_{(1,218)} = 144.3$; $P < 0.001$
Tn - P. citri		4.56±0.22 Aa	4.30±0.24 Aa	3.90±0.19 Aa	
Statistics[b]		$F_{(2,217)} = 213.65$; $P = 0.11$			

*Means followed by the same lower-case letters in a line indicate no differences between adult's sex and larvae. Means followed by the same capital letters on the column do not differ significantly between the individuals of different combinations. The mean comparison was made by the Tukey test ($P < 0.05$).

[a]Statistic from analysis results of factor predator-prey reared on.

[b]Statistic from analysis results of factor adult's sex and 4th instar Larvae.

Table 3: Average (± SE) number of mealybugs consumed by *Cryptolaemus montrouzieri* (*Cm*) and *Tenuisvalvae notata* (*Tn*) reared on *Ferrisia dasylirii* or *Planococcus citri,* during 24 and 48 hours in caged cotton plants in the greenhouse.

Predator-prey reared on	**Prey offered**	**Prey mean consume 24h**		**Statistics [a] (24h)**	**Prey mean consume 48h**		**Statistics [a] (48h)**
		Female	Male		Female	Male	
Cm – F. dasylirii	*F. dasylirii*	4.15±0.22 Aa*	4.30±0.18Aa	$F_{(1,77)}$=37.18; *P*= 0.34	5.95±0.24Aa	4.25±0.20Ab	$F_{(1,77)}$=33.51; *P*= 0.77
Cm - P. citri		4.25±0.24Aa	3.85±0.19Ba		5.30±0.21 Aa	4.70±0.25Ab	
Statistics[b]		$F_{(1,78)}$ =38.07; *P*= 0.06			$F_{(1,78)}$ =33.59; *P*= 0.001		
Cm - F. dasylirii	*P. citri*	5.7±0.11 Aa	4.95±0.11Ab	$F_{(1,77)}$=17.59; *P*< 0.001	7.85±0.18Aa	6.75±0.22Ab	$F_{(1,77)}$ =29.15; *P*< 0.001
Cm - P. citri		5.1±0.18 Ba	3.10±0.18Bb		7.20±0.17Ba	4.35±0.15Bb	
Statistics[b]		$F_{(1,78)}$ =29.9; *P*< 0.001			$F_{(1,78)}$ =50.78; *P*< 0.001		
Tn - F. dasylirii	*F. dasylirii*	3.65±0.21Aa	2.80±0.21Aa	$F_{(1,77)}$=32.81; *P*= 0.81	5.65±0.24Aa	4.65±0.24Ab	$F_{(1,77)}$=33.92; *P*< 0.05
Tn - P. citri		3.35±0.27Aa	2.95±0.17Aa		4.55±0.25Ba	4.05±0.18Ab	
Statistics[b]		$F_{(1,78)}$ =32.93; *P*= 0.05			$F_{(1,78)}$ =39.75; *P*< 0.05		
Tn - F. dasylirii	*P. citri*	3.55±0.14Ba	2.86±0.11Ab	$F_{(1,77)}$=19.73; *P*= 0.08	6.75±0.19 Aa	4.68±0.13Ab	$F_{(1,77)}$ =23.91; *P*= 0.17
Tn - P. citri		4.30±0.15Aa	3.10±0.25Ab		5.55±0.18Ba	4.95±0.18Ab	
Statistics[b]		$F_{(1,78)}$ =22.77; *P*< 0.05			$F_{(1,78)}$ =25.76; *P*< 0.001		

*Means followed by the same lower-case letters in a line indicate no statistical differences and the same capital letters on the column do not differ significantly by the Tukey test (*P*< 0.05).

[a] Statistic from analysis results of factor predator -prey reared on.

[b] Statistic from analysis results of factor adult's

Supplementary Information (SI)

Table S1a: Mean walking time (± SE) spent by adults *Cryptolaemus montrouzieri* (*Cm*) and *Tenuisvalvae notata* (*Tn*) reared on *Ferrisia dasylirii* or *Planococcus citri,* during the prey footprints test.

Predator-prey reared on	**Treatment Area**	**walking time (WT) (seconds)**		**Statistics[a]**
		Female	*Male*	
Cm – F. dasylirii	Untreated A1	193.24±27.43	196.68±17.08	$F_{(1,58)} = 1.22$, $P = 0.27$
	Untreated A2	174.02±26.06	154.13±16.88	
	Statistics[b]	$F_{(1,56)} = 0.06$, $P = 0.79$		
	P. citri	245.02±23.21	273.15±25.18	$F_{(1,58)} = 4.36$, $P < 0.05$*
	Untreated area	207.26±19.05	210.21±21.30	
	Statistics[b]	$F_{(1,56)} = 0.14$, $P = 0.70$		
	F. dasylirii	251.94±14.83	209.73±23.60	$F_{(1,58)} = 1.26$, $P = 0.26$
	Untreated area	222.42±18.14	258.17±26.46	
	Statistics[b]	$F_{(1,56)} = 0.18$, $P = 0.67$		
	P.citri	239.77±26.06	291.74±23.36	$F_{(1,58)} = 4.20$, $P = 0.04$*
	F. dasylirii	257.33±28.32	186.20±24.29	
	Statistics[b]	$F_{(1,56)} = 0.13$, $P = 0.71$		
Cm - P. citri	Untreated A1	194.62±20.49	213.87±22.49	$F_{(1,58)} = 1.43$, $P = 0.23$
	Untreated A2	257.48±16.12	184.12±24.45	
	Statistics[b]	$F_{(1,56)} = 0.009$, $P = 0.92$		
	P. citri	274.68±24.22	259.07±24.54	$F_{(1,58)} = 5.12$, $P < 0.05$*
	Untreated area	227.33±22.54	184.58±18.25	
	Statistics[b]	$F_{(1,56)} = 0.64$, $P = 0.43$		
	F. dasylirii	193.24±27.43	196.68±17.08	$F_{(1,58)} = 5.20$, $P < 0.05$*
	Untreated area	174.02±26.06	154.13±16.88	
	Statistics[b]	$F_{(1,56)} = 0.37$, $P = 0.53$		
	P.citri	227.41±19.87	211.11±26.79	$F_{(1,58)}=1.29$; $P=0.26$
	F. dasylirii	169.94±21.48	240.47±21.21	
	Statistics[b]	$F_{(1,56)} = 0.006$, $P = 0.94$		
Tn - F. dasylirii	Untreated A1	115.32±20.25	166.64±22.74	$F_{(1,58)} = 0.78$, $P = 0.37$
	Untreated A2	179.38±17.63	119.24±22.48	
	Statistics[b]	$F_{(1,56)} = 0.01$, $P = 0.91$		
	P. citri	237.34±33.29	218.49±29.31	$F_{(1,58)} = 6.49$, $P = 0.01$*
	Untreated area	156.89±27.11	173.59±29.29	
	Statistics[b]	$F_{(1,56)} = 0.22$, $P = 0.64$		
	F. dasylirii	178.03±27.03	176.15±29.64	$F_{(1,58)} = 0.71$, $P = 0.39$
	Untreated area	229.32±31.06	207.95±28.80	
	Statistics[b]	$F_{(1,56)} = 0.018$, $P = 0.89$		
	P.citri	173.63±20.17	175.67±23.20	$F_{(1,58)} = 1.32$,

	F. dasylirii	208.43±22.12	206.54±35.75	$P = 0.25$
	Statistics[b]	$F_{(1,56)} = 0.29$, $P = 0.58$		
Tn - P. citri	Untreated A1	199.76±30.21	147.64±27.28	$F_{(1,58)} = 1.56$, $P = 0.21$
	Untreated A2	178.03±28.95	220.36±29.89	
	Statistics[b]	$F_{(1,56)} = 0.32$, $P = 0.57$		
	P. citri	162.03±24.44	221.58±34.25	$F_{(1,58)} = 1.22$, $P = 0.27$
	Untreated area	161.76±29.75	156.67±33.61	
	Statistics[b]	$F_{(1,56)} = 0.36$, $P = 0.55$		
	F. dasylirii	213.85±29.74	178.06±26.20	$F_{(1,58)} = 0.14$, $P = 0.70$
	Untreated area	218.33±34.29	169.26±24.01	
	Statistics[b]	$F_{(1,56)} = 0.24$, $P = 0.62$		
	P.citri	209.63±36.68	171.45±29.52	$F_{(1,58)} = 0.47$, $P = 0.49$
	F. dasylirii	259.44±39.03	183.78±26.01	
	Statistics[b]	$F_{(1,56)} = 0.12$, $P = 0.72$		

[a] Statistic from analysis results of footprint treatment area.

[b] Statistic from analysis results of factor adult's sex

*The variable between the treatment is significant differences ($P<0.05$).

Table S1b. Mean walking distance (± SE) elapsed by adults *Cryptolaemus montrouzieri* (*Cm*) and *Tenuisvalvae notata* (*Tn*) reared on *Ferrisia dasylirii* or *Planococcus citri*, during the prey footprints test.

Predator-prey reared on	**Treatment Area**	**walking distance (WD) (cm)**		**Statistics[a]**
		Female	*Male*	
Cm – F. dasylirii	Untreated A1	75.68±9.82	97.78±8.50	$F_{(1,58)} = 3.03$, $P = 0.08$
	Untreated A2	65.29±8.86	78.15±9.29	
	Statistics[b]	$F_{(1,56)} = 0.14$, $P = 0.70$		
	P. citri	145.08±21.62	140.38±13.05	$F_{(1,58)} = 0.81$, $P = 0.37$
	Untreated area	129.84±15.48	134.26±13.46	
	Statistics[b]	$F_{(1,56)} = 0.48$, $P = 0.49$		
	F. dasylirii	209.35±26.21	188.06±32.51	$F_{(1,58)} = 10.06$, $P = 0.001$*
	Untreated area	224.34±46.41	283.97±60.27	
	Statistics[b]	$F_{(1,56)} = 0.05$, $P = 0.82$		
	P.citri	213.58±42.49	190.65±34.91	$F_{(1,58)} = 0.45$; $P = 0.50$
	F. dasylirii	224.12±60.68	171.22±38.31	
	Statistics[b]	$F_{(1,56)} = 0.45$, $P = 0.50$;		
Cm - P. citri	Untreated A1	140.53±27.47	154.41±28.24	$F_{(1,58)} = 3.62$, $P = 0.06$
	Untreated A2	166.46±25.15	138.85±42.45	
	Statistics[b]	$F_{(1,56)} = 0.04$, $P = 0.83$		
	P. citri	130.08±10.11	136.35±13.04	$F_{(1,58)} = 6.62$, $P = 0.01$*
	Untreated area	109.56±11.35	101.11±11.59	
	Statistics[b]	$F_{(1,56)} = 0.41$, $P = 0.52$		
	F. dasylirii	123.46±19.01	121.48±15.80	$F_{(1,58)} = 1.84$, $P = 0.17$
	Untreated area	123.90±14.92	153.61±30.05	
	Statistics[b]	$F_{(1,56)} = 0.0003$, $P = 0.98$		
	P.citri	120.91±14.79	97.08±12.11	$F_{(1,58)} = 1.96$; $P = 0.16$
	F. dasylirii	88.28±11.62	109.93±10.98	
	Statistics[b]	$F_{(1,56)} = 1.18$, $P = 0.17$		
Tn - F. dasylirii	Untreated A1	50.93±8.21	67.69±8.54	$F_{(1,58)} = 0.56$, $P = 0.45$
	Untreated A2	76.33±7.05	50.00±9.35	
	Statistics[b]	$F_{(1,56)} = 0.08$, $P = 0.76$		
	P. citri	229.03±69.20	227.08±65.13	$F_{(1,58)} = 0.83$, $P = 0.36$
	Untreated area	177.12±63.72	275.58±93.60	
	Statistics[b]	$F_{(1,56)} = 0.66$, $P = 0.42$		
	F. dasylirii	185.71±77.45	241.82±57.69	$F_{(1,58)} = 0.48$, $P = 0.48$
	Untreated area	182.87±51.92	167.15±86.39	
	Statistics[b]	$F_{(1,56)} = 1.31$, $P = 0.25$		
	P.citri	138.64±25.76	19.91±27.46	$F_{(1,58)} = 0.95$; $P = 0.33$
	F. dasylirii	127.53±19.07	112.32±23.77	
	Statistics[b]	$F_{(1,56)} = 0.84$, $P = 0.36$		

Tn - P. citri	Untreated A1	88.22±12.69	69.69±10.71	$F_{(1,58)} = 0.08$, $P = 0.76$
	Untreated A2	67.32±9.24	81.65±9.29	
	Statistics[b]	$F_{(1,56)} = 0.07$, $P = 0.78$		
	P. citri	79.40±13.77	86.17±15.73	$F_{(1,58)} = 1.02$, $P = 0.31$
	Untreated area	59.95±8.86	100.51±28.75	
	Statistics[b]	$F_{(1,56)} = 0.82$, $P = 0.37$		
	F. dasylirii	92.56±16.69	113.64±18.69	$F_{(1,58)} = 1.43$, $P = 0.23$
	Untreated area	84.25±13.09	82.78±12.78	
	Statistics[b]	$F_{(1,56)} = 0.13$, $P = 0.71$		
	P.citri	77.52±13.74	90.02±22.79	$F_{(1,58)} = 0.01$; $P = 0.89$
	F. dasylirii	94.38±16.18	82.27±16.50	
	Statistics[b]	$F_{(1,56)} = 0.10$, $P = 0.74$		

[a] Statistic from analysis results of footprint treatment area.

[b] Statistic from analysis results of factor adult's sex

* The variable between the treatment is significant differences (P<0.05).

Table S1c. Mean walking speed (± SE) used by adults *Cryptolaemus montrouzieri* (*Cm*) and *Tenuisvalvae notata* (*Tn*) reared on *Ferrisia dasylirii* or *Planococcus citri*, during the prey footprints test.

Predator-prey reared on	**Treatment Area**	**Walking speed (WS) (cm/s)**		**Statistics[a]**
		Female	Male	
Cm – F. dasylirii	Untreated A1	0.43±0.03	0.51±0.03	$F_{(1,58)} = 0.12$, $P = 0.72$
	Untreated A2	0.39±0.04	0.49±0.04	
	Statistics[b]	$F_{(1,56)} = 0.64$, $P = 0.42$		
	P. citri	0.55±0.04	0.58±0.03	$F_{(1,58)} = 2.24$, $P = 0.14$
	Untreated area	0.59±0.05	0.64±0.04	
	Statistics[b]	$F_{(1,56)} = 2.53$, $P = 0.11$		
	F. dasylirii	0.79±0.09	1.01±0.14	$F_{(1,58)} = 0.67$, $P = 0.41$
	Untreated area	0.91±0.12	0.97±0.12	
	Statistics[b]	$F_{(1,56)} = 1.26$, $P = 0.26$		
	P.citri	0.86±0.07	0.69±0.10	$F_{(1,58)} = 0.008$; $P = 0.92$
	F. dasylirii	0.71±0.09	0.82±0.10	
	Statistics[b]	$F_{(1,56)} = 0.31$, $P = 0.58$		
Cm - P. citri	Untreated A1	0.78±0.13	0.75±0.11	$F_{(1,58)} = 1.42$, $P = 0.23$
	Untreated A2	0.66±0.06	0.76±0.12	
	Statistics[b]	$F_{(1,56)} = 0.15$, $P = 0.69$		
	P. citri	0.53±0.03	0.56±0.03	$F_{(1,58)} = 0.03$, $P = 0.85$
	Untreated area	0.54±0.04	0.57±0.04	
	Statistics[b]	$F_{(1,56)} = 0.03$, $P = 0.85$		
	F. dasylirii	0.60±0.03	0.71±0.03	$F_{(1,58)} = 9.12$, $P < 0.05$*
	Untreated area	0.54±0.04	0.56±0.03	
	Statistics[b]	$F_{(1,56)} = 0.0003$, $P = 0.98$		
	P.citri	0.49±0.03	0.51±0.03	$F_{(1,58)} = 0.09$; $P = 0.76$
	F. dasylirii	0.49±0.04	0.48±0.05	
	Statistics[b]	$F_{(1,56)} = 0.02$, $P = 0.87$		
Tn - F. dasylirii	Untreated A1	0.41±0.03	0.42±0.03	$F_{(1,58)} = 0.18$, $P = 0.66$
	Untreated A2	0.36±0.03	0.38±0.05	
	Statistics[b]	$F_{(1,56)} = 1.65$, $P = 0.20$		
	P. citri	1.11±0.25	1.03±0.17	$F_{(1,58)} = 0.01$, $P = 0.90$
	Untreated area	1.09±0.22	1.02±0.24	
	Statistics[b]	$F_{(1,56)} = 0.02$, $P = 0.87$		
	F. dasylirii	0.85±0.20	0.91±0.25	$F_{(1,58)} = 0.03$, $P = 0.84$
	Untreated area	0.61±0.09	0.89±0.17	
	Statistics[b]	$F_{(1,56)} = 0.17$, $P = 0.68$		
	P.citri	0.65±0.07	0.57±0.11	$F_{(1,58)} = 4.11$; $P = 0.04$*
	F. dasylirii	0.54±0.05	0.50±0.08	
	Statistics[b]	$F_{(1,56)} = 0.82$, $P = 0.37$		

Tn - P. citri	Untreated A1	0.52±0.05	0.47±0.04	$F_{(1,58)} = 0.003$, $P = 0.95$
	Untreated A2	0.37±0.05	0.40±0.03	
	Statistics[b]	$F_{(1,56)} = 3.40$, $P = 0.06$		
	P. citri	0.43±0.04	0.56±0.09	$F_{(1,58)} = 0.07$, $P = 0.78$
	Untreated area	0.38±0.05	0.52±0.14	
	Statistics[b]	$F_{(1,56)} = 0.66$, $P = 0.79$		
	F. dasylirii	0.50±0.08	0.48±0.06	$F_{(1,58)} = 0.11$, $P = 0.73$
	Untreated area	0.48±0.05	0.59±0.11	
	Statistics[b]	$F_{(1,56)} = 0.51$, $P = 0.47$		
	P.citri	0.37±0.05	0.64±0.12	$F_{(1,58)} = 1.12$; $P = 0.29$
	F. dasylirii	0.38±0.04	0.42±0.05	
	Statistics[b]	$F_{(1,56)} = 0.31$, $P = 0.57$		

[a] Statistic from analysis results of footprint treatment area.

[b] Statistic from analysis results of factor adult's sex

*The variable between the treatment is significant differences (P<0.05).

Table S1d: Mean number of stops (± SE) made by adults *Cryptolaemus montrouzieri* (*Cm*) and *Tenuisvalvae notata* (*Tn*) reared on *Ferrisia dasylirii* or *Planococcus citri,* during the prey footprints test.

Predator-prey reared on	**Treatment Area**	**Number of stops (NS)**		**Statistics[a]**
		Female	Male	
Cm – F. dasylirii	Untreated A1	233.8±37.88	282.64±34.32	$F_{(1,58)} = 0.24$, $P = 0.62$
	Untreated A2	256.0±46.83	220.53±34.16	
	Statistics[b]	$F_{(1,56)} = 0.52$, $P = 0.47$		
	P. citri	239.27±39.95	192.10±32.21	$F_{(1,58)} = 0.64$, $P = 0.43$
	Untreated area	232.48±35.96	147.50±26.64	
	Statistics[b]	$F_{(1,56)} = 0.003$, $P = 0.95$		
	F. dasylirii	265.76±38.52	148.20±28.17	$F_{(1,58)} = 1.54$, $P = 0.22$
	Untreated area	225.76±30.98	233.36±37.17	
	Statistics[b]	$F_{(1,56)} = 0.12$, $P = 0.72$		
	P.citri	200.43±38.73	218.24±38.58	$F_{(1,58)} = 0.06$; $P = 0.79$
	F. dasylirii	130.37±25.78	119.51±23.14	
	Statistics[b]	$F_{(1,56)} = 0.34$, $P = 0.56$		
Cm - P. citri	Untreated A1	133.07±29.51	279.65±34.41	$F_{(1,58)} = 0.97$, $P = 0.32$
	Untreated A2	229.55±41.33	237.86±34.21	
	Statistics[b]	$F_{(1,56)} = 0.15$, $P = 0.69$		
	P. citri	163.64±38.13	263.73±35.32	$F_{(1,58)} = 0.53$, $P = 0.46$
	Untreated area	218.62±46.67	229.43±36.75	
	Statistics[b]	$F_{(1,56)} = 0.16$, $P = 0.69$		
	F. dasylirii	164.17±35.81	153.06±38.84	$F_{(1,58)} = 5.67$, $P = 0.01$*
	Untreated area	184.55±36.17	226.10±44.09	
	Statistics[b]	$F_{(1,56)} = 0.02$, $P = 0.88$		
	P.citri	361.61±35.81	198.24±39.81	$F_{(1,58)} = 16.56$; $P < 0.001$*
	F. dasylirii	194.60±27.41	220.37±38.05	
	Statistics[b]	$F_{(1,56)} = 0.05$, $P = 0.81$		
Tn - F. dasylirii	Untreated A1	186.86±40.27	325.23±44.72	$F_{(1,58)} = 0.51$, $P = 0.47$
	Untreated A2	287.76±36.94	252.93±53.07	
	Statistics[b]	$F_{(1,56)} = 0.06$, $P = 0.90$		
	P. citri	223.5±45.83	193.03±35.33	$F_{(1,58)} = 0.72$, $P = 0.39$
	Untreated area	198.8±43.03	163.56±34.90	
	Statistics[b]	$F_{(1,56)} = 0.002$, $P = 0.96$		
	F. dasylirii	204.53±33.78	242.27±37.41	$F_{(1,58)} = 0.25$, $P = 0.62$
	Untreated area	233.46±43.57	162.51±38.91	
	Statistics[b]	$F_{(1,56)} = 0.24$, $P = 0.62$		
	P.citri	199.37±34.71	223.93±39.60	$F_{(1,58)} = 1.39$; $P = 0.24$
	F. dasylirii	230.53±35.35	148.72±32.08	

	Statistics[b]	$F_{(1,56)} = 0.003$, $P = 0.95$		
Tn - P. citri	Untreated A1	193.37±38.33	182.30±39.88	$F_{(1,58)} = 1.55$, $P = 0.21$
	Untreated A2	180.34±41.50	250.17±43.23	
	Statistics[b]	$F_{(1,56)} = 0.007$, $P = 0.93$		
	P. citri	287.62±46.03	226.43±47.57	$F_{(1,58)} = 8.10$, $P < 0.05$*
	Untreated area	190.58±33.00	122.86±37.76	
	Statistics[b]	$F_{(1,56)} = 0.06$, $P = 0.79$		
	F. dasylirii	199.78±39.66	253.34±41.21	$F_{(1,58)} = 1.36$, $P = 0.24$
	Untreated area	162.83±39.48	207.48±30.44	
	Statistics[b]	$F_{(1,56)} = 0.006$, $P = 0.93$		
	P.citri	156.89±40.71	159.23±33.22	$F_{(1,58)} = 0.58$; $P = 0.44$
	F. dasylirii	142.27±37.32	224.40±39.561	
	Statistics[b]	$F_{(1,56)} = 0.12$, $P = 0.72$		

[a] Statistic from analysis results of footprint treatment area.

[b] Statistic from analysis results of factor adult's sex

*The variable between the treatment is significant differences (P<0.05).

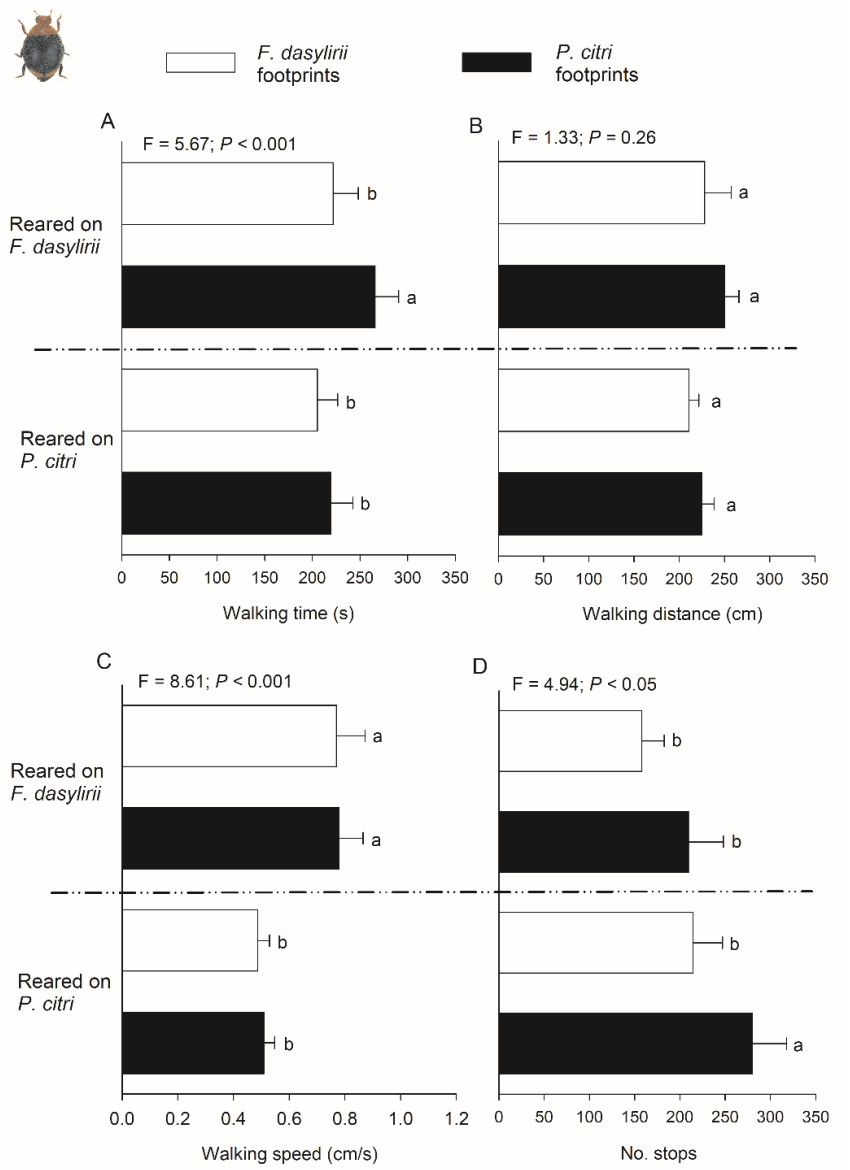

Figure 1. Walking time (A), walking distance (B), walking speed (C), and the number of stops (D), for *Cryptolaemus montrouzieri* reared on different prey (*Ferrisia dasylirii* and *Planococcus citri*) in partially treated arenas with the footprint of each prey (Test 2). The statistics value at the top of each variable corresponds to the differences across the four combinations, analyzed by MANOVA. Means followed by the same letter do not differ statistically among themselves by Tukey test ($P > 0.05$), where means of four treatments (Predator populations and prey offered) were compared.

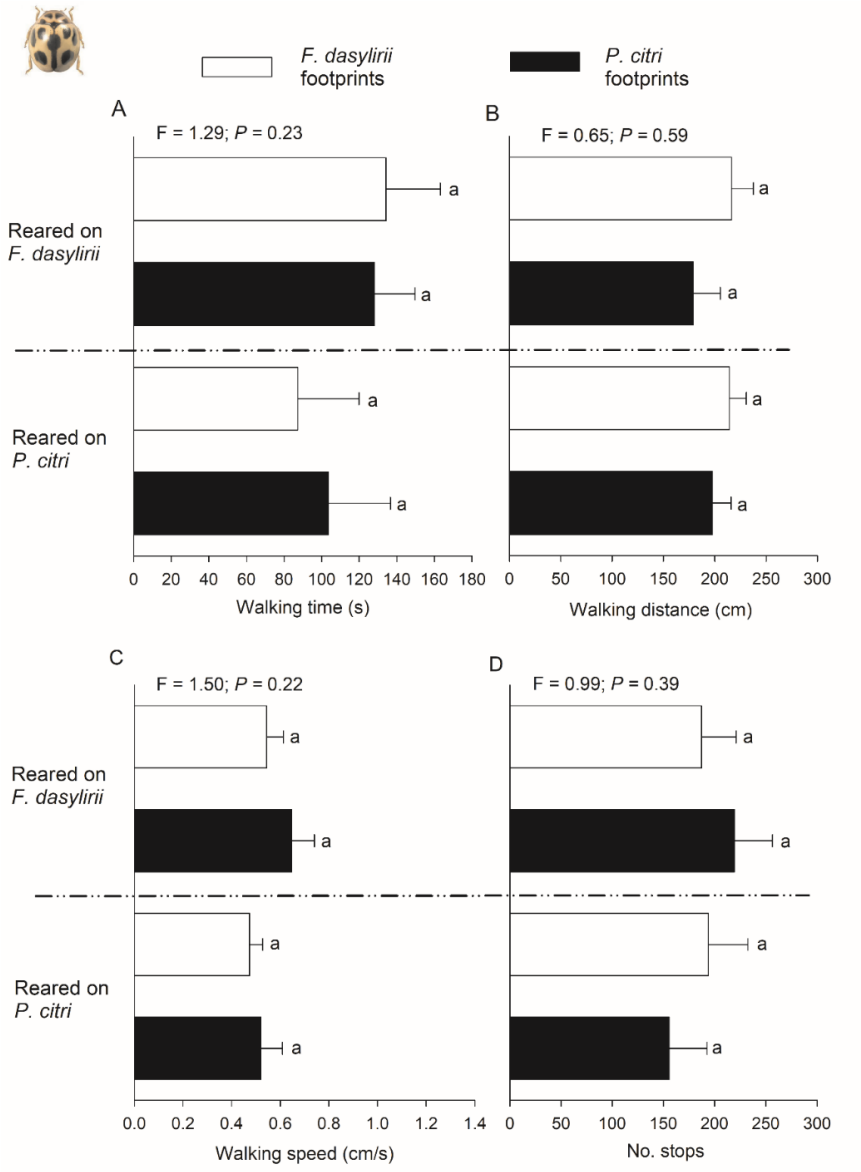

Figure 2. Walking time (A), walking distance (B), walking speed (C), and number of stops (D), for *Tenuisvalvae notata* reared on different prey (*Ferrisia dasylirii* and *Planococcus citri*) in partially treated arenas with the footprint of each prey (Test 2). The statistics value at the top of each variable corresponds to the differences across the four combinations, analyzed by MANOVA. Means followed by the same letter do not differ statistically among themselves by Tukey test ($P > 0.05$), where means of four treatments (Predator populations and prey offered) were compared.

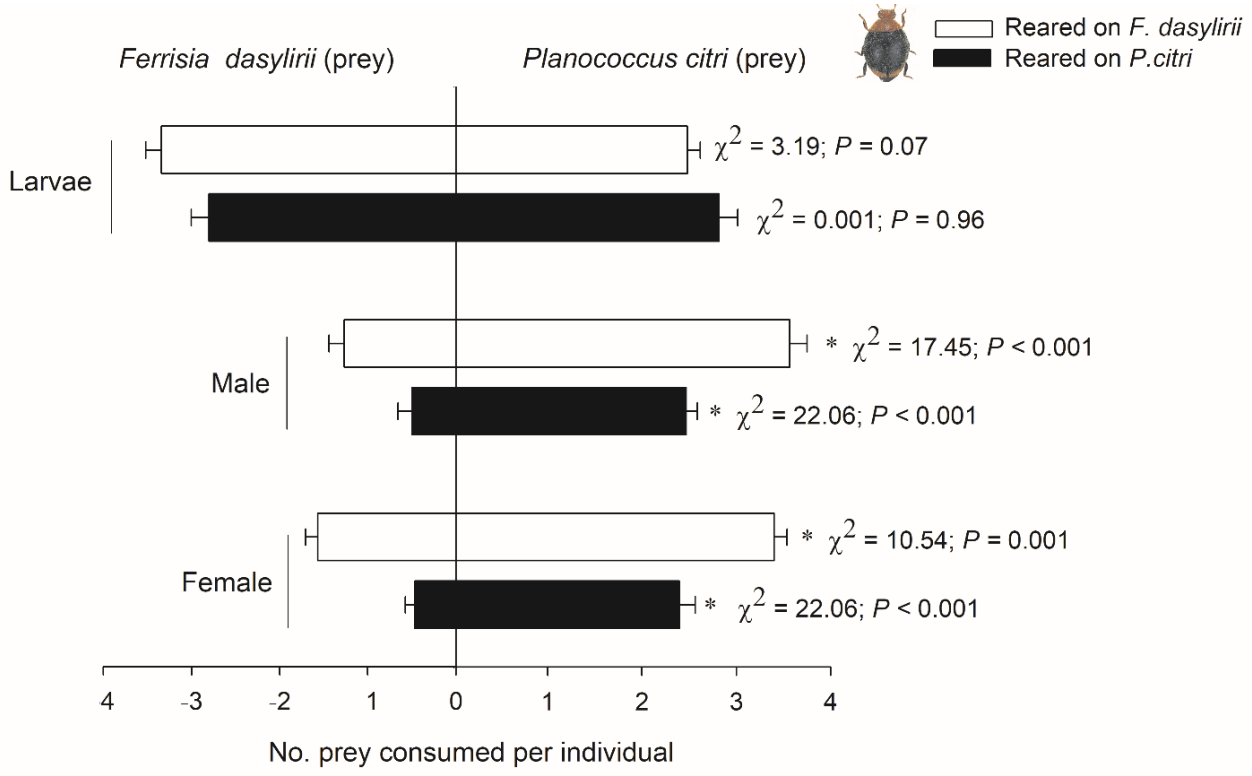

Figure 3. Mean number of mealybugs consumed (+SE) in laboratory by adults and larvae of *Cryptolaemus montrouzieri* reared on different prey (*Ferrisia dasylirii* and *Planococcus citri*) on a two-way choice test. Asterisks indicate significant asymmetry for that pair-wise comparison.

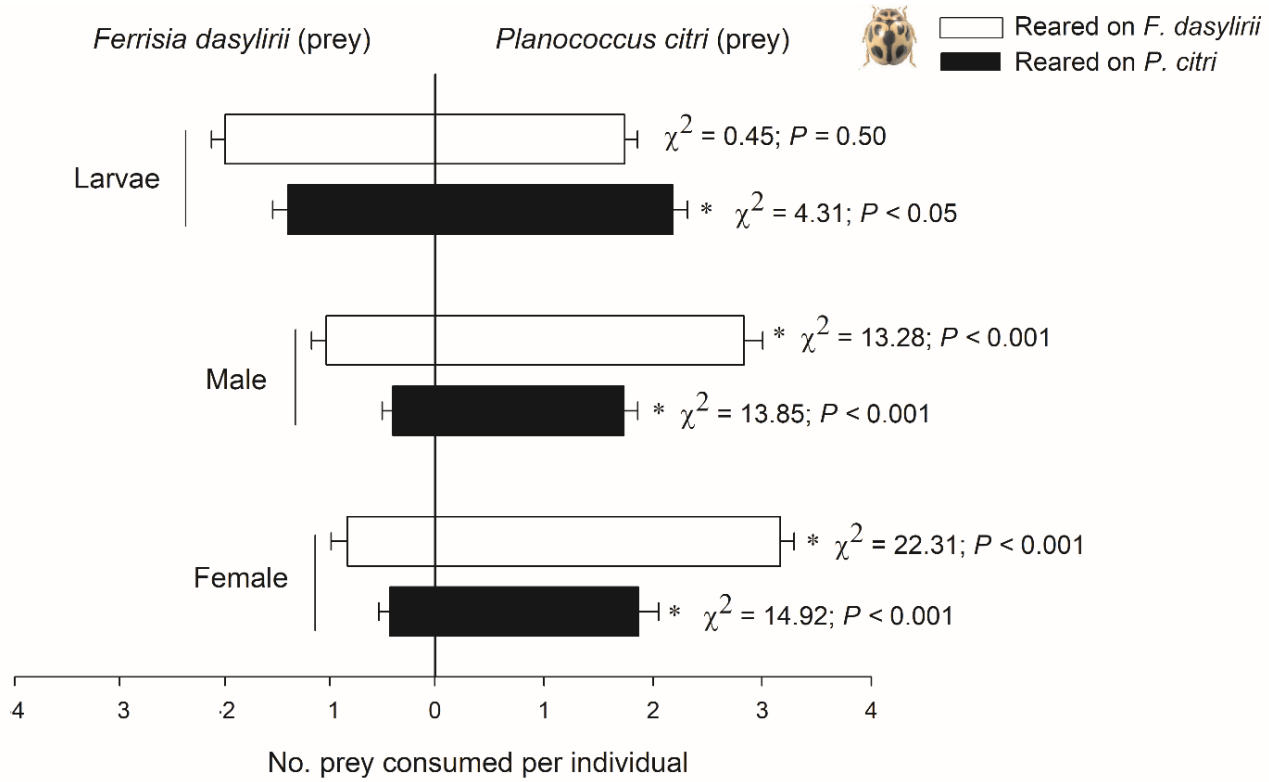

Figure 4. Mean number of mealybugs consumed (+SE) in laboratory by adults and larvae of *Tenuisvalvae notata* reared on different prey (*Ferrisia dasylirii* and *Planococcus citri*) on a two-way choice test. Asterisks indicate significant asymmetry for that pair-wise comparison.

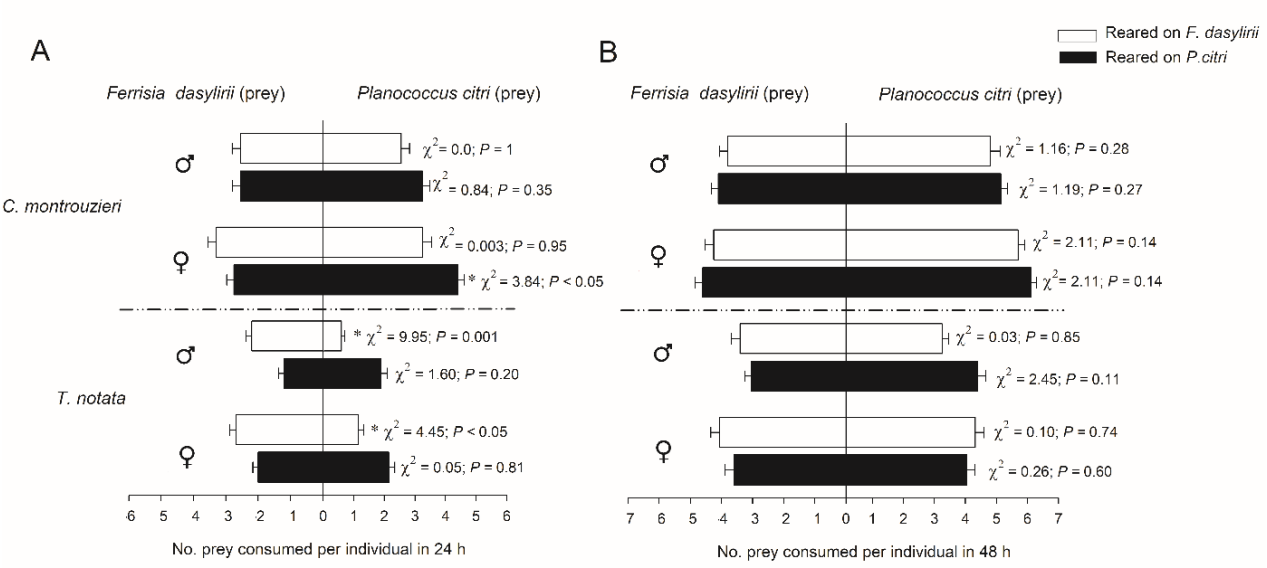

Figure 5. Preference of mealybugs consumed (Mean +SE) during A) 24 hours and B) 48 hours on cotton by adults of *Cryptolaemus montrouzieri* and *Tenuisvalvae notata* that have been reared from two species of mealybugs (*Ferrisia dasylirii* and *Planococcus citri*) (*Free-choice test*). Asterisks indicate significant asymmetry for that combination of sex predators - prey.

CAPÍTULO 3

METABOLIC COSTS AND CHEMICAL PROFILES OF WAX PRODUCTION IN *Cryptolaemus montrouzieri* MULSANT AND *Tenuisvalvae notata* (MULSANT) (COLEOPTERA: COCCINELLIDAE)

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METABOLIC COSTS AND CHEMICAL PROFILES OF WAX PRODUCTION IN *Cryptolaemus montrouzieri* MULSANT AND *Tenuisvalvae notata* (MULSANT) (COLEOPTERA: COCCINELLIDAE)

ABSTRACT – The lady beetles *Tenuisvalve notata* and *Cryptolaemus montrouzieri* are important predators of mealybugs (Hemiptera: Pseudococcidae). Similar to the prey, these lady beetles produce wax filaments that cover their body during the larval stage. It has been hypothesized that lady beetle body wax chemical profiles are similar to their prey as i) a mechanism of camouflage, and ii) conveying protection to the lady beetle larvae against aphid-tending predatory ants. Here, we tested this hypothesis for the predators *T. notata* and *C. montrouzieri* and two mealybug prey species, *Ferissia dasyrilii*, and *Planococcus citri*. We evaluated the influence of feeding on cuticular chemistry during predator development and identified possible metabolic costs associated with wax production. Cuticular wax samples were analyzed by GC-MS and GC-FID. Also, the metabolic cost linked to wax production was evaluated in 4th instar larvae of the two predators when subjected to body wax removal from 0 to 4 times. Results showed that predator body wax profiles are not similar to the chemical profile of prey body wax. There was a metabolic cost associated with wax removal; predators (male and female) showed a significant reduction in adult body weight when wax was removed. This suggests reallocation of energy to wax replacement instead of growth. In addition, we detected effects of wax removal on fecundity and egg viability. Our results do not support the hypothesis that predators mimic the cuticular wax composition of prey as a means of camouflage.

KEY WORDS: Biological control, body wax, coccinellids, cuticular hydrocarbons, metabolism cost, reproduction.

CUSTO METABÓLICO E PERFÍS QUÍMICO DA PRODUÇÃO DE CERA EM *Cryptolaemus montrouzieri* MULSANT E *Tenuisvalvae notata* (MULSANT) (COLEOPTERA: COCCINELLIDAE)

RESUMO – As joaninhas *Tenuisvalve notata* e *Cryptolaemus montrouzieri* são importantes predadores de cochonilhas-farinhentas (Hemiptera: Pseudococcidae). Similar às presas, estas joaninhas produzem filamentos de cera que cobrem o corpo durante a fase larval. Existe a hipótese que a composição química da cera das joaninhas é semelhante àquela de suas presas como i) mecanismo de camuflagem, e ii) proteção das larvas contra formigas predadoras protocooperantes de pulgões. Nesse estudo nós testamos esta hipótese para os predadores *T. notata* e*C. Montrouzieri* e duas espécies de cochonilhas, *Ferissia dasyrilii* e *Planococcus citri*. Foi avaliado a influência do alimento durante o desenvolvimento na composição química cuticular do predador, e identificado um possível custo metabólico associado à produção de cera. As amostras de cera cuticular foram analisadas através da CG-EM e CG-FID. Também, o custo metabólico relativo a produção de cera foi avaliado em larvas de 4th instar dos predadores sujeitos à remoção da cera de 0 a 4 vezes. Os resultados mostraram que os perfis químicos da cera dos predadores não são similares aos perfis de cera das presas. Houve um custo metabólico associado à remoção de cera; predadores (machos e fêmeas) tiveram uma redução significativa do peso corporal do adulto quando a cera foi removida. Isso sugere realocação de energia para recompor a cera do corpo ao invés de crescimento. Adicionalmente, houve efeito da remoção de cera na fecundidade e viabilidade de ovos. Nossos resultados não suportam a hipótese que estes predadores mimetizam a composição da cera cuticular como forma de camuflagem

PALAVRAS-CHAVE: Controle biológico, coccinellídeos, cera corporal, hidrocarbonetos cuticulares, custo metabólico, reprodução.

Introduction

Species that feed on the same kind of food or are in the same trophic level often experience niche overlap (Bruno *et al.* 2003). Interacting species occupying the same ecological niche evolve adaptations to be able to detect cues associated with shared resources and exploit these resources in ways that minimize competition. Resource cues can be chemical (volatile or contact-based), tactile, visual (color and shape), or auditory (sounds or vibrations). Organisms rely on combinations of such cues to locate and identify resources, such as food or prey. Thus, cues indirectly mediate many interactions among species (Bell 1990).

Many studies have explored perception of plant cues by insect herbivores and the ways plant cues mediate herbivore behavior and competition (Jermy 1984). A rich body of work also shows that higher trophic levels, particularly specialist parasitoids, can perceive and respond to cues associated with prey, as well as signals produced by plants in response to herbivore damage. These cues and signals are the mechanisms that modulate host-parasitoid interactions, and the ability of parasitoids to perceive these stimuli is the result of evolutionary adaptations (Vinson 1976, Price *et al*. 1980). Adaptations for cue and signal perception are as important for these specialized organisms as morphological and physiological adaptations evolved to overcome host defenses (Waage 1978, Strand & Vinson 1982).

While the evolutionary adaptations of specialized insects are well studied, comparatively less is known about cue-driven interactions involving insect predators, even though they are important regulators of herbivore communities (Whitcomb 1981). Unlike parasitoids, most predatory insects are polyphagous or oligophagous, suggesting a less intimate and less co-evolved relationship with the prey they feed on (Tauber & Tauber 1987). However, more

generalist insect predators will still evolve adaptations to maximize foraging, prey capture, and consumption (Strand & Obrycki 1996).

Among insect predators, the coccinellids (e.g., lady beetles) are outstanding due to their contribution to pest management worldwide (Michaud 2012). Lady beetles are predators in the larval and adult stages and feed on a wide number of prey species, especially whiteflies, aphids, mites, and mealybugs (Obrycki & Kring 1998). Even though these insects tend to be generalist predators, particular species may specialize on certain prey groups. This is the case for *Cryptolaemus montrouzieri* and *Tenuisvalve notata*, which effectively control Pseudococcidae (Ghafoor *et al.* 2011) and mealybugs in Brazil (Ferreira *et al.* 2020). *C. montrouzieri* was introduced to Brazil, while *T. notata* is native to the Neotropical region (Dreyer *et al.* 1997). Among the mealybug species consumed by these lady beetles, *Ferrisia dasylirii* and *Planococcus citri* are important pests of citrus, cotton, papaya, vegetables, ornamentals, and other high-value crops (Ben-Dov 2005, Miller *et al.* 2012).

Mealybugs have a high reproductive rate and rapidly colonize plant tissues. This rapid colonization also facilitated a mutualism with ants, which tend mealybugs to harvest sugary fecal secretions and, in doing so, often provide protection from predators (Way 1963). This ant-mealybug interaction presents a problem for foraging lady beetles. If ant tending is enough of an impediment to foraging, we would expect that lady beetles may have evolved adaptations to access prey while avoiding detection by mealybug tending ants (Jiggins *et al.* 1993). Previous studies have shown that the wax production by some lady beetle larvae in the genus *Scymnus* (e.g., *S. nigrinus* (Kugelann) and *S. interruptus* (Goeze)), can serve as a sort of camouflage to enable entry into congregations of prey while avoiding predation or parasitism (Völkl & Vohland 1996). The same way, as the body wax is known as a defense mechanism used by mealybugs

against predators and parasitoids (Gullan & Kosztarab, 1997), the coccidophagous lady beetles use body wax as an adaptation to improve predation efficiency upon mealybugs (Seago *et al.* 2011). Thus, the body wax in lady beetles works like an indirect defense strategy with physical camouflage, providing an opportunity for larvae to enter prey colonies more easily, and improving survival as reported for *Scymnus louisianae* (Schwartzberg *et al.* 2010). Nevertheless, ants are adapted to recognize visual and chemical stimuli from individuals and respond with appropriate behaviors, such as foraging in response to mealybug-associated stimuli, or defensive behaviors in response to predator intrusion (Jackson & Morgan 1993). This suggests that the strategy used by lady beetle larvae must be more than visual camouflage (Völkl 1995), and could potentially include mimicry of its own prey, as is evident for the *Scymnus* species.

Chemical mimicry will be favored by selection if the benefits, in terms of resource acquisition and eventual fitness, outweigh the metabolic costs of producing the chemicals and/or the potential costs of being perceived as prey (e.g., intraguild predation). These trade-offs are better known as the resource allocation hypothesis, which suggests that a higher investment of energy toward survival comes with a corresponding decrease in reproduction (Boggs 2009). Thus, it is hypothesized that if wax production in lady beetle larvae is a defense mechanism of high metabolic cost, it could divert resources away from reproduction (Eisner 1994). This was observed in the lady beetle *Scymnus nubilus* (Mulsant), which also produces body wax in the larval stage. When this wax was removed from late instars, inducing wax replenishment, it caused a reduction in adult body weight and increased the larval developmental period (Pacheco *et al.* 2021).

To build on this initial finding, we further examined the metabolic costs of wax production through wax removal experiments. We hypothesized that there are metabolic costs of

wax production and that these costs can be detected as changes in the development, survival, and reproduction of the lady beetles. Also, we investigated the chemical profiles of the wax produced by the lady beetles *T. notata* and *C. montrouzieri*, as well as the profiles of wax produced by their mealybug prey. Our goal was to determine if these species also engage in chemical mimicry of their prey, possibly as a means of avoiding attack by tending ants.

Methods and Materials

Prey species. Colonies of *F. dasylirii* and *P. citri* were reared on pumpkins var. "Jacarezinho", obtained from the local market using previously established protocols (Sanches & Carvalho 2010). The colony of *F. dasylirii* has been kept in the laboratory for about a decade and was originally obtained from *Opuntia* plants in the Semiarid region of Pernambuco State (Dormentes County: 09°04'15'' S, 40°19'54'' W) (Barbosa *et al.* 2014). The colony of *P. citri* was collected from Anonaceae plants in the Agreste region of Pernambuco State (Chã-Grande County: 8.25710°S and 35.49386°W, 496 m altitude) and has been kept in the same laboratory since 2016 (Pacheco da Silva *et al.* 2019). Pumpkins were washed and dried, placed in plastic trays (30 x 45 x 4 cm) lined with a paper towel, and infested on the petiole with gravid mealybugs, originating from the stock colony. The average time to complete the infestation of a pumpkin is about 30 days, after which infested pumpkins were used to feed lady beetle colonies. Colonies were kept under the temperature of 25 ± 2 °C, relative humidity of 60 ± 10%, and photoperiod of 12:12 h (L:D).

Predators. Adults of *C. montrouzieri* and *T. notata* were kept under the same conditions as the mealybugs. They were placed in acrylic cages (40x25x20cm), with circular lateral openings,

covered with a fine mesh to allow ventilation inside the boxes. The bottom of the boxes was lined with a paper towel, upon which one infested pumpkin was offered to the predators, following Barbosa *et al.* (2014). Two different populations of each lady beetle species, *C. montrouzieri* and *T. notata*, were established on two different prey species for at least eight generations, *F. dasylirii* and *P. citri*, respectively. Therefore, the four lady beetle populations were: i) *C. montrouzieri* fed *F. dasyrilii*, ii) *C. montrouzieri* fed *P. citri*, iii) *T. notata* fed *F. dasyrilii* and, iv) *T. notata* fed *P. citri*.

Effect of wax removal and replacement on lady beetle fitness. To evaluate the cost of wax production on fitness of *C. montrouzieri* and *T. notata*, 4th instar larvae were subjected to either wax removal or not (control). Wax removal was done with the help of a puro marta paint bush (nº 00), 30 seconds was necessary for this procedure. Insects were subjected to one of four removal treatments: one removal (1x), two removals (2x), three removals (3x), or four removals (4x), with a 24 h interval in between removals. This time interval proved to be enough for wax regeneration by larvae. Lady beetle larvae used in bioassays originated from colonies reared on the respective prey, *F. dasylirii* and *P. citri*. Therefore, we had 20 treatments, formed of the 4 lady beetle populations (each lady beetle reared on the two preys) and the five (5) wax removal treatments, each treatment had 30 replicates. After wax removal, larvae continued to be fed in the same diet (prey) they originated from until pupation. Period of development from 4th instar larva to adult emergence, larval survival, adult body weight, and adult survival were measured.

Preliminary results showed that higher metabolic costs were evident on treatments of 2x and 4x wax removal. Therefore, adults emerged from those respective treatments were used to evaluate the effects of wax removal on female fecundity and fertility. For this experiment there

were 12 treatments (comprised of four populations of lady beetles and 0x, 2x, and 4x wax removal), each with 40 replicates, 20 males and 20 females, further used in the fecundity and fertility tests. For this, females were paired with males originating from the same treatment (n = at least 20/treat) for 24 h in Petri dishes (3.5 cm diam) lined with a small piece of paper towel (0.5 cm2) as a substrate for oviposition. After this period, males were separated from the females which remained in the same Petri dishes. Nymphs of either *F. dasylirii* or *P. citri* were offered in abundance to them as a food source according to the prey species they had been reared on during larval development. Female fecundity and egg viability were measured for 60 consecutive days.

Data for the time until adult emergence and adult body weight were subjected to deviance analysis (ANODEV), then analyzed using a General Linear Model (GLM) with Gaussian error distribution, followed by a contrast analysis to separate the means ($P<0.05$) as described by Crawley (2007). Additionally, female and larval survival curves, according to their respective prey and wax removal treatment, were subjected to survival analysis using Kaplan–Meier (K-M) estimation, and the survival curves Pairwise comparisons of variables levels were compared by Log Rank (Mantel-Cox), using the car, ggplot2, survival and the pairwise_survdiff() function of survminer (Wickham 2016, Fox & Weisberg 2019, Kassambara *et al.* 2020, Therneau 2022).

Female fecundity and egg viability were subjected to Shapiro-Wilk normality test to check for ANOVA assumptions. Date of female fecundity and fertility (number of eggs hatched) were not normally distributed and were subjected to deviance analysis (ANODEV) Data were analyzed using a General Linear Model (GLM) with Poisson error distribution. Dispersion was corrected by a Quasipoisson distribution, followed by a residual analysis to verify the error distribution and model construction. We then performed a contrast analysis to separate the means ($P<0.05$) as

described by Crawley (2007). These analyses were performed using the software R 4.2.1 (R Development Core Team 2022).

Chemical analysis of body wax from lady beetles and mealybugs.

Extracts. We collected body wax from the 4th instar larvae of the lady beetles *C. montrouzieri* and *T. notata*, fed on each prey species, *F. dasylirii* or *P. citri*. We also collected wax from third instar mealybug nymphs reared on pumpkins and reared for one generation on cotton plants (cv. IMA 2106GL). Fifteen individuals of each species and age were used to collect wax in each treatment as follows: i) Larvae of *C. montrouzieri* fed on *F. dasylirii*; ii) Larvae of *C. montrouzieri* fed on *P. citri*; iii) Larvae of *T. notata* fed on *F. dasylirii*; iv) Larvae of *T. notata* fed on *P. citri*; v) Nymphs of *F. dasylirii* reared on pumpkins; vi) Nymphs of *P. citri* reared on pumpkins; vii) Nymphs of *F. dasylirii* reared on cotton; and viii) Nymphs of *P. citri* reared on cotton. Wax extracts were obtained by washing the larvae/nymphs in each treatment with 100 µl of hexane for 10 minutes. Next, the extracts were filtered in glass wool placed inside a micropipette tip, which worked as a "funnel". A 10µl sample was collected from each extract and placed in a glass vial with 10 ng/µl of the internal standard n-heptadecane to a final concentration of 1.6ng/µl.

Quantification of cuticular hydrocarbons. For quantitative analysis, wax extracts were analyzed by gas chromatography coupled to a flame ionization detector (GC-FID) [GC2010 Shimadzu, Kyoto, Japan] equipped with a non-polar stationary phase column (30 m × 25 µm × 25 mm; Rtx-1, Agilent J&W, Santa Clara, CA, USA). The oven was programmed to 100° C for 0.10 minutes, then to 270° C at 10° C per min, held for 63 minutes. Aliquots of 1 µL of each sample (n=6

replicates) were injected using the splitless mode, with the inlet at 250º C, and helium as the carrier gas (30.3 cm/sec). Data were collected using GC Solution (version 2.32.00). The quantification was done comparing the area of the n-heptadecane with the areas of all compounds in the chromatogram profile.

Identification of cuticular hydrocarbons. Extracts were analyzed using a Shimadzu GCQP-2010 Ultra mass spectrometer (GC-MS; GCQP-2010 Ultra, Shimadzu Corp., Kyoto, Japan) equipped with a non-polar column of stationary phase (30 m × 25 μm × 25 mm; Rtx-1MS; RESTEK, Bellefonte, PA, USA), a splitless injector, and helium as the carrier gas (39.9 cm/sec). Ionization was by electron impact (70 eV, (40-400 m/z, source temperature at 270 °C). Aliquots of 1 μL of each sample were injected at 250 °C using the same temperature program as in GC-FID analysis. A standard of alkanes (C7–C40; Sigma-Aldrich) was also injected for retention index calculation (Van Den Dool & Kratz 1963). In the chromatograms, peaks were integrated using GCMS Solution software (version 4.20), and cuticular compounds were identified based on their retention indices and their mass spectra by comparison with NIST libraries.

Seeking to reduce the variables that explain the profile variation between species, the similarity of chemical composition between the body wax of *T. notata, C. montrouzieri, F. dasylirii* and, *P. citri* was qualitatively analyzed by principal component analysis (PCA) using FactoMineR package (Lê *et al.* 2008) with the software R 4.2.1 (R Development Core Team 2022). The hydrocarbons profile (presence and amount of the compound) present on body wax of each species (prey and predator) was subjected to a non-metric multidimensional scaling analysis (NMDS) based on the Bray-Curtis distance index (Bray & Curtis 1957) to calculate the data matrix of pairwise comparisons among samples. Prior to this, data were standardized using a

Wisconsin double standardization, to remove the possible "noise" of the data. The NMDS analyses were performed with R "Vegan" package v 2.5-6. The first analysis includes all the species, testing the null hypothesis of no difference in wax chemical composition between ladybug larvae and mealybugs species, for that we used the permutation multivariate analysis of variance (PERMANOVA) by employing the function 'Adonis' in the 'Vegan' package with the Bray-Curtis similarity measurement and 999 permutations. The second analysis only included the subset of predator-prey combination, to test the null hypothesis that the wax chemical profile of lady beetles is not affected by the prey they fed on. The same analysis was used for testing the hypothesis of no differences in chemical profile of prey (mealybug) reared on two plant hosts, pumpkin and cotton.

Results

Effect of wax production cost on lady beetle fitness- There was a significant effect of wax removal (1x to 4x) on the developmental period from 4th instar larvae to adult emergence in both lady beetle species (*C. montrouzieri,* $F_{(1,284)}$= 132.51; P< 0.0001; *T. notata,* $F_{(1,286)}$= 108.21; P=0.0002). There was no significant difference in the developmental period of males and females emerged after wax removal (*C. montrouzieri,* $F_{(1,283)}$= 0.95; P=0.33; *T. notata,* $F_{(1,285)}$= 106.86; P=0.25). Moreover, for *C. montrouzieri* there was a significant effect of prey species on developmental period of larvae subjected to wax removal (1x-4x) or not (control) ($F_{(1,288)}$= 218.05; P=2.2e^{-16}), with a shorter developmental period of those fed on *F. dasylirii* (Table 1). In contrast, for *T. notata* there was no significant effect of prey species on larval developmental period ($F_{(1,290)}$= 129.57; P=0.52). Regarding survival, there was no effect of wax removal with 100% larval survival on all treatments.

For the adults, wax removal had a significant effect on adult body weight on both lady beetle species (*C. montrouzieri*: $F_{(4,284)}$= 3.75; *P*=0.005; *T. notata*: $F_{(4,286)}$= 22.96; *P*< 0.0001) (Table 1). For *C. montrouzieri* ($F_{(1,283)}$= 86.41; *P*< 0.0001) and *T. notata* ($F_{(1,285)}$= 215.14; *P*< 0.0001) females were heavier than males. Also, there was a significant effect of prey species on adult (male and female) body weight (*C. montrouzieri*: $F_{(1,288)}$= 116.8; *P*< 0.0001; *T. notata*: $F_{(1,286)}$= 71.59; *P*< 0.0001) (Table 2).

Adult (female) survival was affected by the prey species a female had been reared on and the wax removal treatment (Figs. 1 and 2). We detected no effect of 2x wax removal in the larval stage on later survival of *C. montrouzieri* adult females (χ^2=3.7, DF= 3, P = 0.3) (Fig. 1a). In contrast, 2x wax removal caused a significant reduction in *T. notata* female survival (χ^2=10.3, DF= 3, P = 0.02) (Fig. 2a). Moreover, 4x wax removal affected the survival of adult females in both lady beetle species (*C. montrouzieri*: χ^2=9.6, DF= 3, P = 0.02); *T. notata* (χ^2=15.5, DF= 3, P = 0.001) (Figs. 1b and 2b).

Female fecundity was affected by the prey species the lady beetles had been reared on (*C. montrouzieri*: $F_{(1,294)}$= 28.60; *P*<0.0001; *T. notata*: $F_{(1,329)}$= 62.95; *P*<0.0001). Wax removal only affected fecundity for *T. notata* ($F_{(1,323)}$= 27.74; *P*<0.0001). In addition, fecundity was reduced for *T. notata* females when they were mated with males, both fed *F. dasylirii*, and subjected to 2x and 4x wax removal (Table 3). When the prey was *P. citri*, there was also a reduction in *T. notata* fecundity with increasing frequency of wax removal on 4th instar larvae (Table 3). In contrast, we did not detect a reduction in fecundity for *C. montrouzieri* females due to wax removal ($F_{(1,288)}$= 0.88; *P*=0.504) no matter what prey were provided (Table 3).

Regarding egg viability, in *T. notata* there was a significant effect of prey species ($F_{(1,329)}$= 55.71; *P*<0.0001) and wax removal treatments ($F_{(1,323)}$= 23.06; *P*<0.0001) (Table 3). For *C.*

montrouzieri, egg viability was affected only by the prey species they were reared on ($F_{(1,294)}$= 13.23; P<0.0001), not by the wax removal treatments ($F_{(1,288)}$= 0.64; P=0.69). In addition, females of *T. notata* and *C. montrouzieri* fed *P. citri* had lower egg viability than those fed on *F. dasylirii* (Table 3).

Chemical analysis of body wax from lady beetles and mealybugs- Overall, 37 compound were identified in the body wax of lady beetle larvae and mealybug nymphs (each peak area exceeding 0.5% of total peak area) (Table 4). The principal components analysis (PC1 = 35% and PC2 = 21%) did not group the extracts of lady beetle species with the profile of their prey. The PCA graph shows that there is no relationship between the compounds detected and the species from which compounds originated (Fig. 3). Body wax blends were able to be differentiated visually via NMDS ordination (Stress = 0.08, R^2 = 0.969) (Fig. 4), but there was no significant difference between all the species combination tested ($F_{(7,38)}$=32.84 ; P= 0.0001) (Table 5a).

Feeding on different species does not generate changes in the chemical profile in the predator body wax, but the chemical profile of each predator is different (Table 5b). However, when the mealybugs feed on a different host plant, the wax chemical profile changes, in the individuals reared on cotton were show more compounds and differences in the proportion of those, as is illustrated in the NMDS (Fig 2.) and, the PERMANOVA analysis (Table 5c). Also, the chemical profile of mealybugs is different for each species ($F_{(1,20)}$= 12.99; P= 0.001) (Table 5c).

Discussion

In this study we found that the body wax present on larvae of the lady beetles, *T. notata* and *C. montrouzieri*, is metabolically costly to produce. For both lady beetle species, wax removal affected their developmental times, suggesting a reallocation of energy resources to restore this defense mechanism. Wax removal also affected other performance metrics (e.g., fecundity) in a species-specific manner. These findings suggest that body wax serves an important function for the insects, as production continued despite costs. Similar to our study, prior work with *Scymnus* also found that the reallocation of energy resources due to wax removal during the larval stage was costly, causing a reduction in adult body weight (Pacheco *et al.* 2021).

Even though the adult fecundity was affected by wax removal, we did not find any effects on larval survival. 100% of 4th instar larvae survived after wax was removed from their body. This suggests that wax production is an adaptive trait for the coccinellid species studied here. Wax can be removed naturally by predators as they attempt to attack and consume coccinellid larvae. Replacement of the wax in response to removal ensures that this defense is replenished after it acts to deter predation (DeWitt *et al.* 1998, Johansson & Mikolajewski 2008). If this process of replacement significantly affected larval survival in a negative way, it is unlikely that it would be maintained. Our finding of effects of wax removal on larval development rate suggests there are some metabolic costs, but these are balanced with other physiological needs. However, the cost reflected in this trait may involve a decrease in the larvae survival in field conditions. In nature the regeneration period of the wax, can extend the food deprivation time (Phoofolo *et al*. 2008), and increase the risk of attack by another insect (Schwartzberg *et al.* 2010).

Wax removal also had effects that carried on into the adult stage. These effects varied depending on the species. For *C. montrouzieri,* there was no effect of wax removal on female fecundity and egg viability. In contrast, for *T. notata* there was a negative effect on that traits, regardless of larval sex (male or female) subjected to wax removal. In addition, this effect was directly related to the number of times wax was removed, with a higher impact for insects subjected to 4x wax removal. These different variations may be related to the life-history strategies of each species, since fixed defense traits are associated with constant contact with predators, environment pressure, and the genetic pool of each species (Johansson & Mikolajewski 2008, Johnson & Belk 2020). Thus, the differential response between *C. montrouzieri* and *T. notata* suggests that, as in species of the genus *Tribolium* (used commonly as a biological model), the response to external stimuli depends on the population properties and their plasticity in adapting to interactions on the field (Pointer *et al*. 2021). For example, relative to *C. montrouzieri, T. notata* has a reduced size and impressive longevity (450 days under optimal conditions (Dreyer *et al.* 1997)). As a result of these life history traits, wax removal for this species may incur greater trade-offs, resulting in lower fecundity as a cost. The extent of this trade-off may also depend on timing. Overall results suggest that wax removal on 4th instar larvae by itself is not a factor that will affect the fitness of the lady beetles. There is variation in coccinellids, and energy reallocation can have different effects depending on the number of times wax was removed, and the species subjected to it.

Prey type and quality can also influence predator traits. In this study, larval developmental time was shorter and adult predator weight was higher when lady beetles were reared on *F. dasylirii*. There was also a significant effect of prey type on the developmental time of *C. montrouzieri*, but not on *T. notata*. That we observed these effects is not surprising, as a prior

study on both species also documented prey-driven differences in performance metrics (Ferreira *et al*. 2020). Similarly, other Coccinellidae also shows the same kind of variation in prey effects on developmental times, as reported for *Hippodamia convergens* (Michaud 2000, Ponsonby 2009), *Cheilomenes sexmaculata* and *Oenopia conglobata* (Thompson 1999, Mirhosseini *et al.* 2015). However, we did not observe an interaction between wax removal and prey type on predator traits.

Body wax can function directly, as a physical barrier (Völkl & Vohland 1996), and indirectly, as a chemical defense mechanism to avoid tending-ant recognition (Delabie 2001, Hayashi *et al.* 2016, Schwartzberg *et al*. 2010). Our removal assays confirmed that wax is metabolically costly to produce, and therefore, likely serves an important function. This could include acting as a physical deterrent to predation. We hypothesized that another important function for body wax of these two species is chemical mimicry of prey. This hypothesis is based on prior research with Scymninae, which found that wax production is used as camouflage allowed the predator to approach the prey more easily, and also changes in response to the type of prey consumed (mealybugs) (Pope 1979, Michaud 2005, Seago *et al.* 2011).

Despite these prior findings for Scymninae, our results not showed an overlap in chemical profiles between lady beetles and their prey. This suggests that for the two species studied here, the wax may function primarily as a direct defense against predation, and most likely does not double as a chemical camouflage. The chemical profile of body wax produced by each predator species is different and it is not related to the prey species it fed on. The PCA analysis showed differences in the chemical profiles of each lady beetle species, and no compound in common characterizes both profiles of predator and prey. Similarly, the results of the NMDS analyses suggest that the chemical profiles of species are different, with the excellent representation

model determined by the level of stress (Stress = 0.08) and confirmed by the results of the PERMANOVA analysis. Thus, the chemical composition of body wax in *C. mountrozieri* and *T. notata* seems to be related to species/population traits and not to prey type. This could be justified by the oligophagous feeding habit of these lady beetle species that prey on different species of Pseudococcidae and may even prey on other resources such as pollen and nectar (Sloggett & Majerus 2000). Therefore, there is no co-dependence between predator and prey species strong enough to create or select for chemical mimicry. In contrast, other species that have a strong co-dependence do show certain similarities in their chemical profiles, such as the physogastric thermophilous rove beetle and its host *Constrictotermes cyphergaster*, indicating a chemical disguise that allows the rove beetle to live as inquiline in the termite colony (Rosa *et al.* 2018).

Another factor disfavoring chemical mimicry among the predator species may be the variation in the body wax chemical profiles of mealybug prey. We found that the chemical profile of the prey species varied depending on the host plants they fed during development. For an oligophagous predator, this means that there is no one body wax composition "target" for selection to favor. On the contrary, on herbivory insect, the switch between enviorments (eg. host plants) promovote changes in the CHC profile, as an adaptive phenotypic plasticity response, that can drive many evolutionary processes like species selection, or the establishment of reproductive isolation barriers between populations (Chung & Carroll 2015, Otte *et al.* 2018). This kind of response has been reported to scale insects, like *Drosicha stebbingiia* (Green) (Hemiptera: Monophlebidae), where their population differ in at least 13 compounds in the cuticular wax composition, when were develop in two different hosts, *Tectona grandis* L.f. and *Pongamia pinnata* L. (Ahmad *et al.* 2020).

Overall, our results suggest that wax production is an important anti-predator strategy for larvae of *C. montrouzieri* and *T. notata*. Individuals subjected to wax removal (e.g., confrontation with predators) can restore all body wax coverage in 24 hours. Both species experience metabolic costs due to wax regeneration, although the magnitude of these costs differs between the two species, with the commercially available *C. montrouzieri* having the fewest costs. This suggests that wax production confers an advantage, and that this trait is maintained populations of each species. Prior studies demonstrate that body wax can facilitate the escape of lady beetle larvae from the attack of tending ants because ants will have wax stuck in their mouth parts. In the time it takes ants to remove the wax from mouthparts, lady beetle larvae can escape the area (Schwartzberg *et al*. 2010, Liere & Perfecto 2014). While this direct physical defense is likely functioning for the two species studied here, our chemical analysis of wax alongside the wax produced by mealybug prey shows no overlap between predator and prey wax compositions. This finding does not support the original hypothesis that both species use a chemical mimicry strategy to resemble prey and avoid predation by mealybug tending ants. Body wax in lady beetles is not related to the species of prey they feed and is species-specific. In contrast, the chemical profile of mealybugs depended on the host plant. Our study shows there are important differences among lady beetle groups in how wax functions to deter predation and facilitate foraging (e.g., chemical mimicry in *Scymnus posticalis* but not *C. montrouzieri* and *T. notata*). Future work should further explore, how species-level differences in metabolic costs of wax production shape efficacy as biological control agents, especially in the context of Pseudococcidae-ant mutualisms.

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Table 1. Mean (±SEM) development time (days) of IV instar larvae to adult emergence of *Cryptolaemus montrouzieri* and *Tenuisvalvae notata* after different wax removal treatments (number of times) depending on the prey species they were reared on, *Ferrisia dasyrilii* and *Planococcus citri*.

Predator/prey	**Wax removal treatment***				
	Control (no removal)	**1x**	**2x**	**3x**	**4x**
C. montrouzieri					
F. dasylirii	5.03 ±0.23Ab	4.03± 0.03Ac	5.16 ±0.14Ab	8.00±0.001Aa	5.00 ±0.001Ac
P. citri	7.71 ±0.38Bb	6.00 ±0.001Bc	7.17 ± 0.07Bb	9.03 ± 0.33Ba	5.04 ± 0.04Bc
T. notata					
F. dasylirii	7.56±0.16Ab	8.00 ± 0.19Ab	9.85±0.31Aa	9.66±0.70Aa	9.76±0.32Aa
P. citri	8.90±0.31Ab	8.10±0.23Ab	9.33±0.16Aa	10.62±0.29Aa	9.00±0.41Aa

*Value followed by the same capital letter (when present) in the columns are not significantly different between the prey species. Treatments followed by the same lowercase letters indicated no differences between them. Means were compared by a contrast analysis ($P<0.05$).

Table 2. Mean (±SEM) weight (mg) of males and females of *Cryptolaemus montrouzieri* and *Tenuisvalvae notata* reared on *Ferrisia dasylirii* and *Planococcus citri*, subjected to various combinations of wax removal treatments.

Predator/prey	**Wax removal treatment***				
	Control (No removal)	**1x**	**2x**	**3x**	**4x**
C. montrouzieri ♀					
F. dasylirii	12.56 ±0.19Aa	11.69± 0.17Ab	11.65 ±0.18Ab	11.53±0.39Ab	11.54±0.22Ab
P. citri	10.22 ±0.42Ba	10.44 ±0.38Ba	10.53 ± 0.36Ba	9.96 ± 0.37Ba	9.47 ± 0.19Ba
C. montrouzieri ♂					
F. dasylirii	10.82±0.23Aa	10.39 ±0.11Aa	10.29± 0.16Aa	10.42±0.18Aa	10.05 ± 0.15Aa
P. citri	9.76 ±0.35Ba	9.37 ± 0.29Ba	9.39±0.31Ba	8.68±0.20Ba	9.15 ± 0.35Ba
T. notata ♀					
F. dasylirii	6.70±0.13Aa	5.98 ± 0.22Ab	5.89±0.11Ab	5.64±0.12Ac	5.47±0.19Ac
P. citri	5.51±0.18Ba	5.19±0.15Bb	4.88±0.23Bb	5.07±0.18Bb	4.23±0.19Bc
T. notata ♂					
F. dasylirii	4.95 ±0.12Aa	4.79±0.14Ab	4.56±0.21Ab	4.22±0.16Bc	3.92±0.16Ac
P. citri	4.49 ± 0.16Ba	4.18±0.12Ba	4.20±0.17Ba	4.12±0.17Aa	3.17±0.36Bb

*Value followed by the same capital letter (when present) in the columns are not significantly different between the prey species. Treatments followed by the same lowercase letters indicated no differences between them. Means were compared by a contrast analysis ($P<0.05$).

Table 3. Reproductive traits of *Cryptolaemus montrouzieri* and *Tenuisvalvae notata* subjected to wax removal treatments and reared on two different prey, *Ferrisia dasyrilii* and *Planococcus citri*.

Predator/prey	Wax removal treatment*						
	Control	**2X-female**	**2X-male**	**2X-(female-male)**	**4X-female**	**4X-male**	**4X-(female-male)**
***C. montrouzieri* Fecundity**							
F. dasylirii $^{n.s.}$	105.8 ±11.53A	84.09 ± 10.89A	108.0 ±12.74A	104.58 ± 11.33A	123.09 ±10.99A	120.36 ±10.53A	98.92 ±11.67A
P. citri $^{n.s.}$	91.00 ±12.35B	87.09 ±7.30B	67.0 ± 7.46B	85.62 ± 7.03B	74.25 ± 9.04B	72.24 ± 8.14B	66.10 ± 6.55B
***C. montrouzieri* Egg viability**							
F. dasylirii $^{n.s.}$	88.22 ±10.65A	71.48± 9.66A	91.70±11.24A	81.75±9.98A	103.95 ±70.00A	103.55±9.61A	81.63±10.64A
P. citri $^{n.s.}$	72.20 ±11.22B	82.70 ±7.16B	62.66± 7.08B	81.95 ± 6.84B	70.0 ± 8.81B	64.33± 7.82B	61.25± 6.50B
***T. notata* Fecundity**							
F. dasylirii	121.15 ± 6.38Aa	88.41 ± 7.93Ab	61.50 ± 7.36Ac	70.48 ± 6.08Ac	76.48 ± 7.88Ac	48.85 ± 9.56Ad	47.95 ± 9.06Ad
P. citri	89.10 ± 7.61Ba	45.43 ±4.67Bb	28.10 ± 5.86Bc	39.76 ± 3.63Bb	34.86 ± 4.47Bc	34.30 ± 5.65Bc	32.52 ± 4.52Bc
***T. notata* Egg viability**							
F. dasylirii	94.55 ± 4.72Aa	66.90 ± 5.64Ab	45.91 ± 5.96Ab	52.66 ± 4.69Ab	59.86 ± 6.95Ab	37.65 ± 7.41Ac	37.38 ± 7.32Ac
P. citri	64.30 ± 6.15Ba	39.90 ± 4.22Bb	19.90 ± 3.67Bc	36.05 ± 3.18Bb	29.38 ± 3.69Bc	25.75 ± 3.82Bc	29.95 ± 4.06Bc

$^{n.s}$ Indicated that the treatments on the row are not significantly different between them.

*Value followed by the same capital letter (when present) in the columns are not significantly different between the prey species. Treatments followed by the same lowercase letters indicated no differences between them. Means were compared by a contrast analysis ($P<0.05$).

Table 4. Peaks of possible chemical compounds from body wax of larvae of lady beetles and preys. Cm_F= *Cryptolaemus* reared on *Ferrisia*, Cm_P = *Cryptolaemus* reared on *Planococcus,* Tn_P = *Tenuisvalvae* reared on *Planococcus,* Tn_F= *Tenuisvalvae* reared on *Ferrisia,* P_Pumpkin= *Ferrisia* reared on pumpkin, P_Pumpkin= *Planococcus* reared on pumpkin, F_Cotton= *Ferrisia* reared on cotton, P_Cotton= *Planococcus* reared on cotton.

Peak Number	Retention time	Kovats iu	Species
1	14.44	2073	Tn_F; Tn_P
2	14.53	2083	Tn_F; Tn_P
3	15.67	2209	Cm_F
4	15.86	2231	Tn_P
5	16.35	2288	Tn_F; Tn_P
6	16.44	2301	Tn_F; Tn_P; Cm_F; Cm_P
7	16.50	2308	Tn_F; Tn_P
8	16.91	2355	Cm_F
9	17.39	2413	Cm_F; Cm_P
10	17.95	2497	Cm_P
11	18.15	2493	Cm_P
12	18.77	2562	F_Cotton
13	19.00	2585	Tn_F
14	19.14	2591	Tn_F; Tn_P
15	19.35	2616	Cm_F; Cm_P
16	20.08	2679	P_Cotton
17	20.30	2690	Cm_F; Cm_P
18	20.41	2707	Cm_F
19	21.16	2760	Cm_F;F_Cotton; P_Cotton
20	21.55	2789	Cm_F; Cm_P
21	22.75	2862	Cm_P; P_Cotton
22	23.09	2882	P_Cotton
23	23.40	2890	Tn_F; Tn_P; Cm_F; Cm_P
24	24.64	2960	Tn_F; Tn_P; Cm_F; Cm_P; F_Pumpkin; P_Pumpkin; F_Cotton; P_Cotton
25	25.44	2988	Cm_F
26	26.03	3022	Tn_F
27	27.04	3061	Cm_P; P_Cotton
28	28.04	3089	Cm_F, Cm_P; F_Cotton

29	29.96	3159	Cm_F; Cm_P; F_Cotton; P_Cotton
30	32.33	3227	Tn_F; Tn_P
31	33.69	3261	Cm_F; Cm_P; P_Cotton
32	35.19	3288	Tn_F; Tn_P; Cm_F; F_Pumpkin; P_Pumpkin; F_Cotton; P_Cotton
33	39.93	3394	F_Cotton
34	42.53	3437	F_Cotton
35	46.43	3488	F_Cotton; P_Cotton
36	64.07	3689	F_Pumpkin;F_Cotton; P_Cotton
37	70.59	3753	Tn_F; Tn_P; Cm_F; Cm_P; F_Pumpkin; F_Cotton;

Table 5. PERMANOVA based on Bray-Curtis similarity lady beetle (predator) and mealybugs (prey) fed on different food sources. Factors: Species = 8 populations (2 predators reared on 2 preys, and 2 preys reared on two host); Plant-hosts = pumpkin and cotton. df = degrees of freedom; SS= Sums of square; Ms= Mean square.

a.	***PERMANOVA for all the species together***					
	df	**SS**	**MS**	**F. model**	**R^2**	***P***
Species	7	12.322	1.760	32.837	0.858	<0.0001***
Residual	38	2.037	0.053		0.141	
Total	45	14.359			1.000	
b. PERMANOVA for the lady beetles reared on two mealybug species						
	df	**SS**	**MS**	**F. model**	**R^2**	***P***
Lady beetle	1	3.740	3.740	39.837	0.633	0.001***
Mealybug (prey)	1	0.195	0.195	2.077	0.033	0.099
Residual	21	1.9718	0.0939		0.334	
Total	23	5.907			1.000	
c. PERMANOVA for mealybugs reared on two plant hosts						
	df	**SS**	**MS**	**F. model**	**R^2**	***P***
Mealybug	1	0.952	0.952	12.995	0.202	0.001***
Host	1	2.285	2.285	31.187	0.485	0.001***
Residual	20	1.465	0.073		0.331	
Total	22	4.702			1.000	

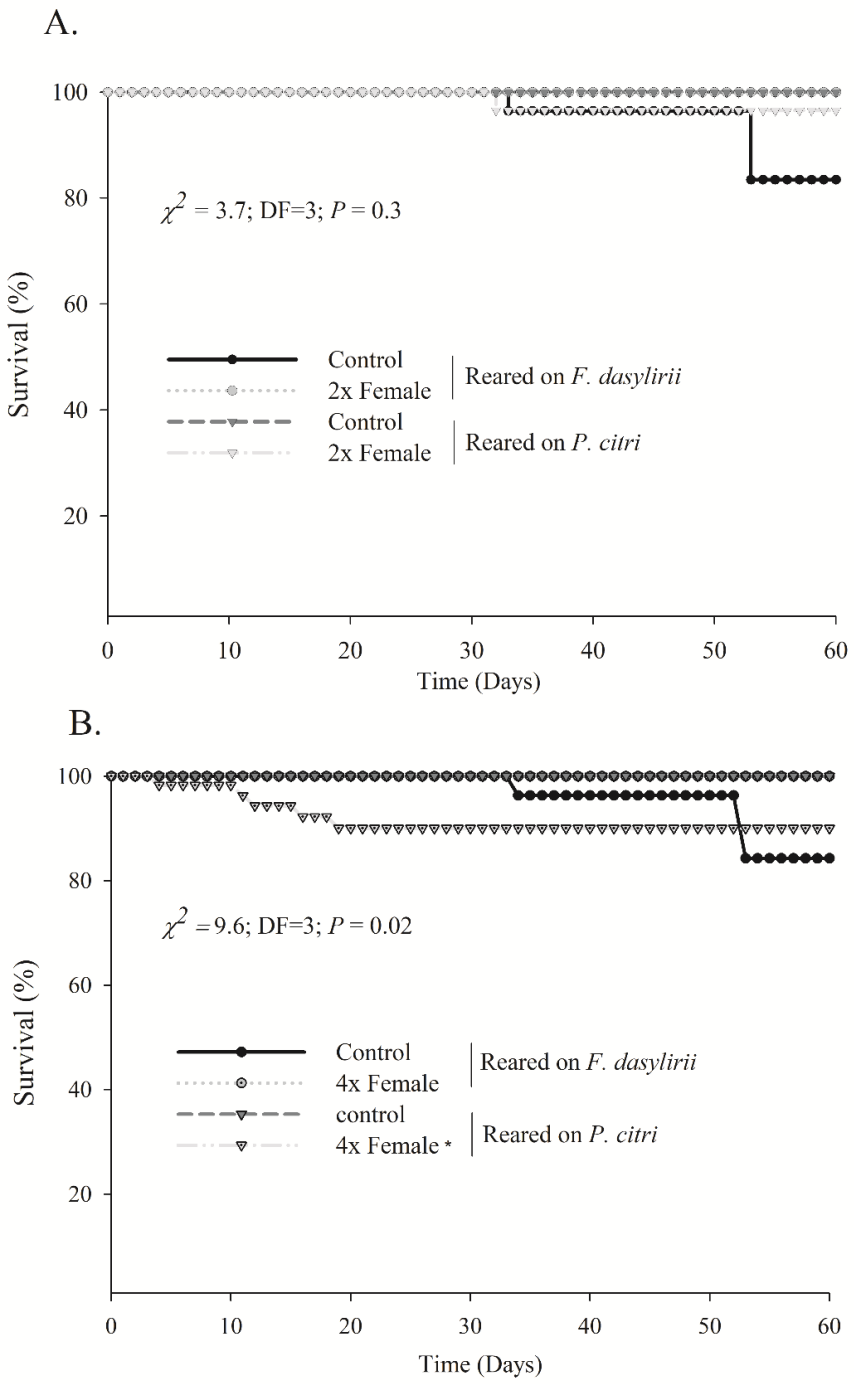

Figure 1. Survival curves of *C. montrouzieri* females emerged from two wax removal treatments [A. 2 times (2x); B. 4 times (4x)] and reared on two different prey species, *Ferrisia dasyrilii* and *Planococcus citri*, during 60 days observation period. Data was subjected to survival analyses using the Kaplan–Meier estimators' log-rank Test ($\alpha = 0.05$).

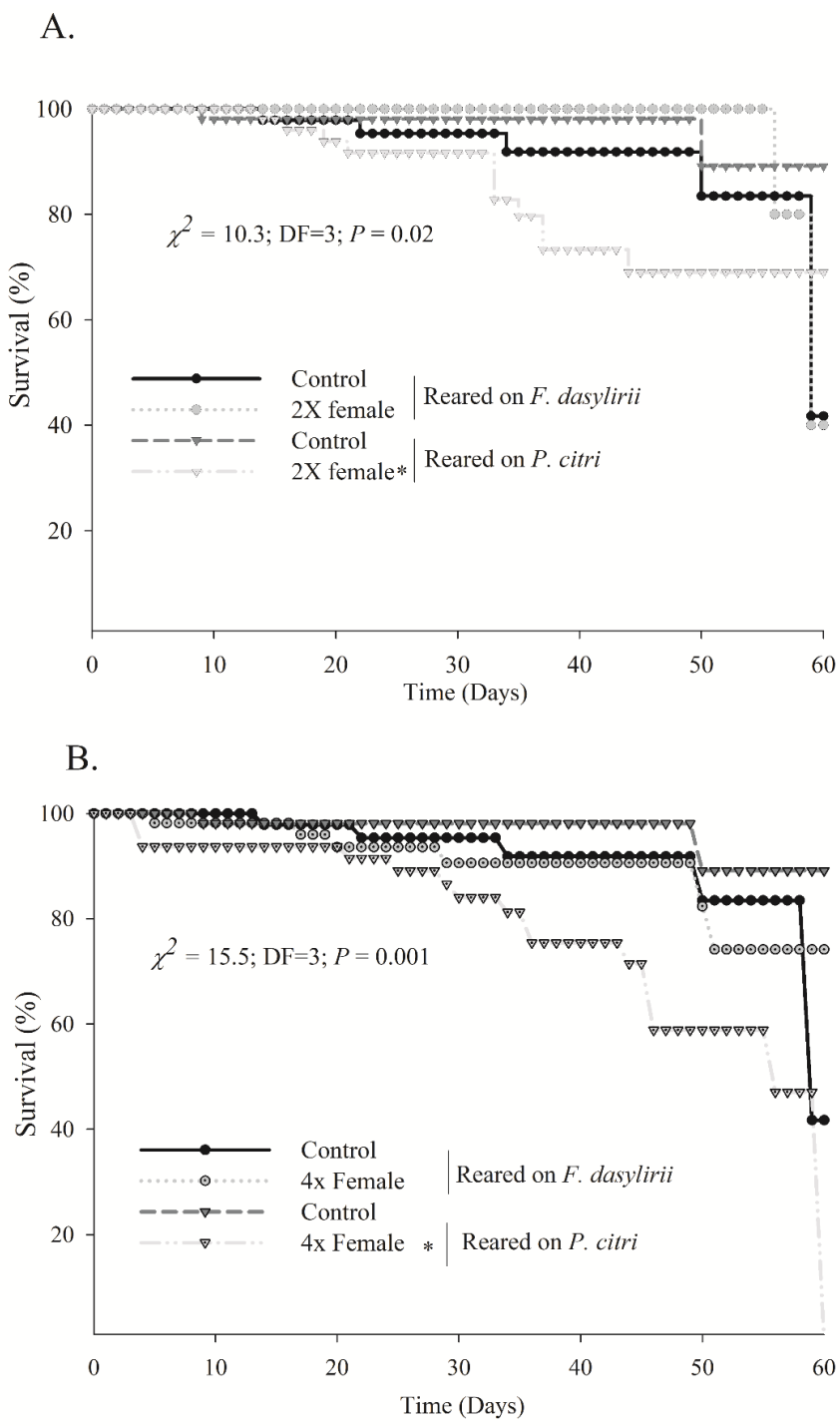

Figure 2. Survival of *T. notata* females emerged from two wax removal treatments [A. 2 times (2x); B. 4 times (4x)] and reared on two different prey species, *Ferrisia dasyrilii* and *Planococcus citri*, during 60 days observation period. Data was subjected to survival analyses using the Kaplan–Meier estimators' log-rank Test ($\alpha = 0.05$).

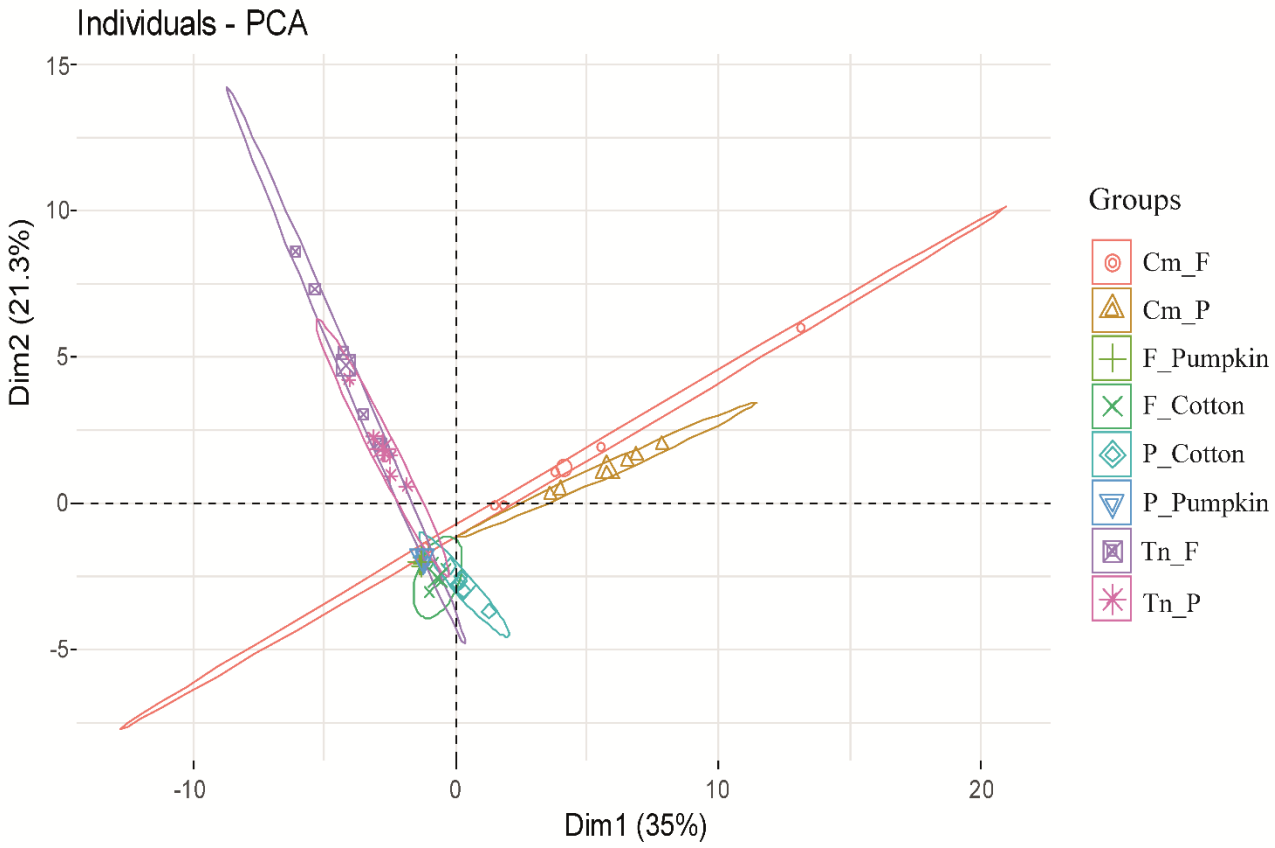

Figure 3. Principal component analysis (PCA) analysis was based on the chemical profiles of the body wax of eight populations of lady beetles and mealybugs. Legend = Cm_F= *Cryptolaemus montrouzieri* reared on *Ferrisia dasylirii;* Cm_P= *C. montrouzieri* reared on *Planococcus citri.* Tn_F= *Tenuisvalvae notata* reared on *F. dasylirii;* Tn_P= *T. notata* reared on *P. citri.* F_Pumpkin= *F. dasylirii* on pumpkin; P_Pumpkin= *P. citri* on pumpkin; F_Cotton= *F. dasylirii* on cotton; P_Cotton= *P. citri* on cotton

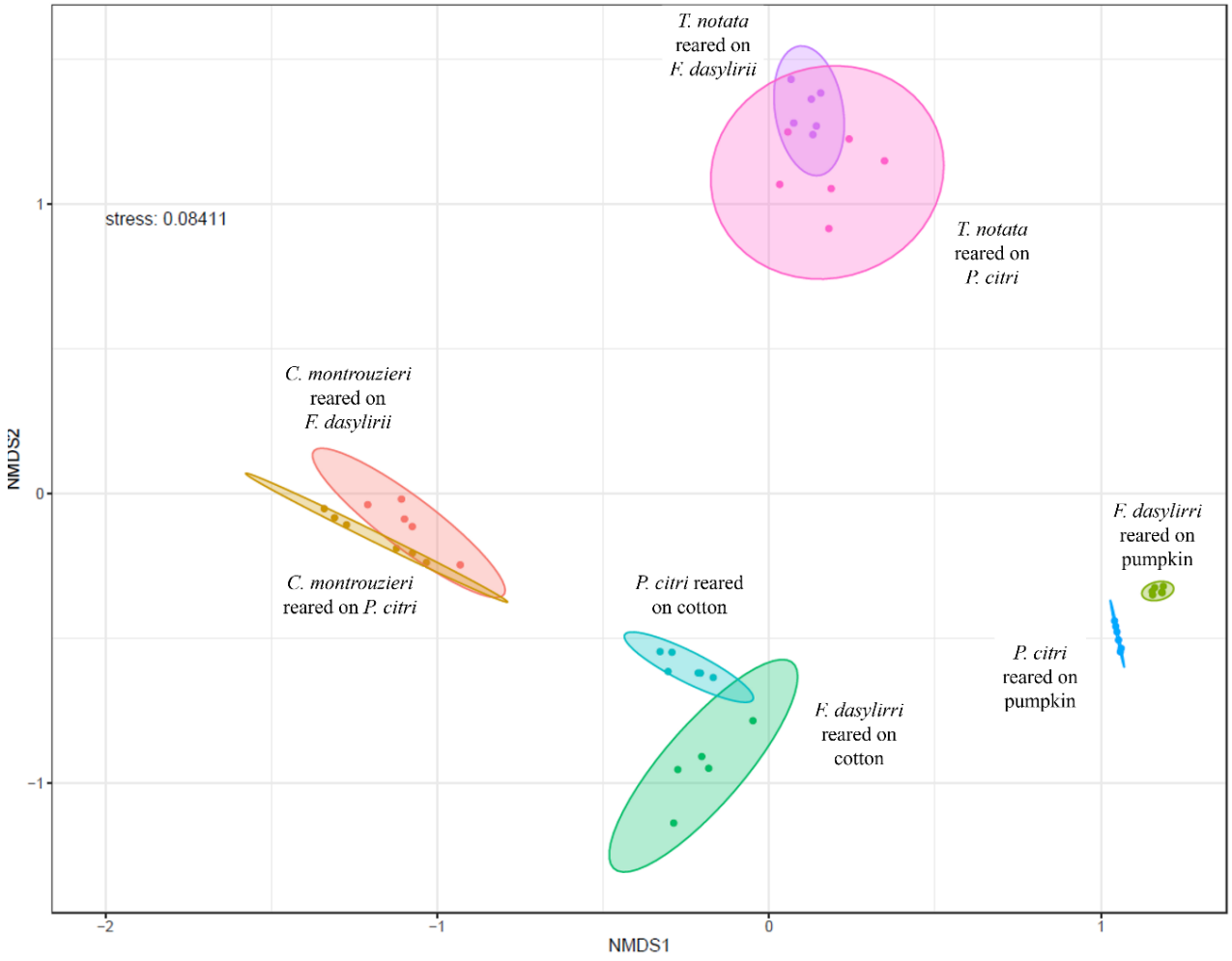

Figure 4. Chemical profiles of body wax of lady beetles (*Cryptolaemus montrouzieri* and *Tenuisvalvae notata*) and prey (*Ferrisia dasylirii* and *Planococcus citri*), reared on different plant host, respectively. Data were visualized using a non-metric multidimensional scaling ordination based on a Bray-Curtis dissimilarity matrix and plotted on two dimensions (stress = 0.08, R2 = 0.969); each species had 95% confidence ellipses around class centroids.

CAPÍTULO 4

CONSIDERAÇÕES FINAIS

No controle biológico os processos de produção e liberação de inimigos naturais são fundamentais para o sucesso da aplicação deste tipo de método de controle de pragas. Fatores como a eficiência de forrageamento, estabilidade no comportamento de predação/parasitismo e boa seletividade de presas/hospedeiros são algumas das características de um agente de controle biológico efetivo. Portanto, as condições de criação massal e dos protocolos padronizados de criação em insetários são fatores importantes a analisar antes da liberação do organismo no campo.

Entre as espécies usadas para controle biológico, encontramos a joaninha *Cryptolaemus montrouzieri* Mulsant, amplamente comercializada e introduzida a nível mundial, para o controle de cochonilhas farinhentas. No entanto, devido às limitações fitossanitárias que podem implicar a introdução de novas espécies, é preconizado o uso de espécies nativas efetivas para produção massal e liberação em campo. Nesse contexto, encontramos a *Tenuisvalvae notata* (Mulsant), nativa de América do Sul e também reportada como voraz predador de cochonilhas. No entanto, para produzir uma espécie de inimigo natural em larga escala e liberá-la em novas áreas, é necessário entender os fatores que podem afetar o desenvolvimento e o comportamento de predação, e como estes estão condicionados pelas interações interespecíficas predador - presa. Nesse estudo foi constatado que em condições padronizadas de criação, com desenvolvimento geracional sobre uma única presa, as espécies de joaninas coccidófagas, *C. montrouzieri* e *T. notata*, não apresentam modificações na capacidade de predação quando são oferecidas presas

diferentes as que foram criados. Ambas espécies de joaninhas responderam aos voláteis emitidos pelas presas, mas não de forma preferencial e a quantidade de consumo das presas está condicionado à qualidade da presa e não ao condicionamento pré-imaginal. Por outro lado, o estimulo associado ao rastro das presas não parece ser suficiente para desencadear um comportamento de procura pelos predadores.

As interações entre organismos, como é o caso da relação predador-presa, pode levar a expressão de caracteres adaptativos que promovam a permanência das espécies em determinado local. Por isto, as joaninhas coccidófagas desenvolveram estratégias como a produção de cera na fase larval, como uma adaptação que facilita a predação das suas presas. Isto porque as larvas podem entrar nas colônias das presas, as quais também produzem filamentos de cera, e passam despercebido tanto das presas como de outros possíveis predadores, tais como as formigas protocooperantes de insetos sugadores. O nosso estudo mostra, que a produção de cera é um caráter adaptativo fixado nas espécies, *C. montrouzieri* e *T. notata*, e esta produção representa um custo metabólico, que na necessidade de regeneração, se evidencia um aumento do tempo de desenvolvimento das larvas, redução no peso dos adultos e consequentemente no fitness das espécies. Além disso, alguns fatores biológicos como a sobrevivência e fecundidade dos predadores podem variar pelas necessidades nutricionais de cada espécie e pela qualidade da presa. Como se evidencia em *T. notata*, os efeitos de remoção da cera no fitness foram mais expressos.

Contradizendo a hipótese de camuflagem química e semelhança entre composição química da cera de presa e predadores, o perfil químico da cera das espécies foi específico para cada uma delas, sem importar o tipo de presa da qual se alimentaram. Assim, a cera produzida

por estas joaninhas representa uma estratégia de defesa física direta e indireta contra organismos associados a pseudococcídeos, como é o caso das formigas protocooperantes.

Por fim, apesar das joaninhas coccidófagas serem importantes agentes do controle biológico de cochonilhas, poucas são as pesquisas que analisam os mecanismos ecológicos, que mediam a interação com suas presas e favorecem sua presença no campo. Portanto, os resultados obtidos neste estudo podem contribuir para preencher algumas lacunas de conhecimento dessas interações e serve de base para pesquisas futuras a fim de entender os fatores que mediam as interações predador-presa com o ambiente, elementos que serão necessários para garantir o sucesso da liberação de controladores biológicos no campo.