REPRODUÇÃO E DESEMPENHO DA DESCENDÊNCIA EM *Eriopis connexa* (GERMAR) (COLEOPTERA: COCCINELLIDAE)

Por

ALESSANDRA CRISTINA GUEDES DA SILVA

(Sob Orientação do Professor Jorge Braz Torres - UFRPE)

RESUMO

A joaninha *Eriopis connexa* (Germar) está amplamente distribuída nos agroecossistemas, e a ocorrência de populações naturalmente selecionadas para resistência a piretroides a torna um promissor agente de controle biológico, para uso e conservação em programas de manejo de pragas. O presente estudo buscou esclarecer a idade de maturidade sexual, seleção do parceiro sexual e a paternidade dos descendentes nesta espécie. Também, investigou-se a deposição de ovos inférteis em função do fenótipo de resistência a inseticidas e da escassez alimentar; e se o desempenho da espécie sofre influência do canibalismo e da predação intraguilda sobre ovos de *Coleomegilla maculata* DeGeer. Os resultados mostram que a maturidade sexual teve início aos 3 dias após a emergência, com total maturidade aos 5 dias. A maior frequência de cópula ocorreu na primeira hora da fotofase e o fenótipo resistente não afetou a escolha do parceiro sexual. Além disso, a paternidade resulta da mistura de espermatozoides e não depende da ordem de acasalamento. A deposição de ovos inférteis foi semelhante entre os fenótipos suscetível e resistente e independente do tamanho da postura. Ainda, a escassez de presas influenciou a fecundidade, mas não a proporção de ovos inférteis. Fêmeas alimentadas apenas com ovos coespecíficos não tiveram melhor desempenho. Contudo, o canibalismo somado a predação intraguilda de ovos de *C. maculata* melhoraram o desempenho de larvas e adultos. O canibalismo e predação intraguilda foram comuns, porém menos frequentes em alta densidade da presa extraguilda (ex. pulgões), prevalecendo o comportamento afidófago da espécie. Esses resultados sugerem que populações de *E. connexa* suscetíveis e resistentes teriam uma perspectiva simples para a produção de progênie, e que o canibalismo e a predação intraguilda possuem papel relevante como complemento de dieta. Contudo, não houve um total esclarecimento sobre a alta frequência de deposição de ovos inférteis, mesmo em condições supostamente ideais de acasalamento.

PALAVRAS-CHAVE: Reprodução, seleção sexual, história de vida, ovos tróficos, escassez de alimento.

REPRODUCTION AND OFFSPRING PERFORMANCE IN THE *Eriopis connexa* (GERMAR) (COLEOPTERA: COCCINELLIDAE)

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ABSTRACT

The lady beetle *Eriopis connexa* (Germar) is widely distributed in the agroecosystems, and the occurrence of populations naturally selected for resistance to pyrethroids make this species an interesting agent of biological control to be preserved in pest control programs. Due to its importance, this study aimed to define the age of sexual maturity, the selection of sexual partners, and the paternity of descendants. In addition, the oviposition of infertile eggs was investigated as a function of the insecticide-resistant phenotype and food scarcity; and if the fitness of this species is affected by cannibalism, and intraguild predation upon eggs of *Coleomegilla maculata* DeGeer. Results show that the sexual maturity in *E. connexa* started within 3 days of adult emergence, but fully developed after 5 days. The highest frequency of mating occurred within first hour of pairing within photophase, and the phenotype for insecticide resistance did not affect the choice of a mating pair. The progeny paternity results from a mixture of available sperm, and do not depend on mating order. The oviposition of infertile eggs was similar between the susceptible and resistant phenotypes regardless of clutch size. Also, prey scarcity affected fecundity, but not infertile egg laying. Females fed only conspecific eggs did not improve fitness.

On the other hand, egg cannibalism and intraguild predation mixed with standard prey increased larval and adult fitness. Cannibalism and intraguild predation were common but less frequent under the high density of the extraguild prey (e.g. aphids), prevailing the aphidophagous behavior in *E. connexa*. These results suggest that the resistant and susceptible *E. connexa* populations have a simple perspective on progeny production and that egg cannibalism and intraguild predation have an important role as a diet supplement. However, there was not a complete understanding of the frequent oviposition of infertile eggs, even under supposedly optimal mating conditions.

KEY WORDS: Reproduction, sexual selection, life history, trophic eggs, food deprivation.

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“O homem é parte da natureza e sua guerra contra ela inevitavelmente uma guerra contra si mesmo... Temos pela frente um desafio como nunca a humanidade teve, de provar nossa maturidade e nosso domínio, não da natureza, mas de nós mesmos.”

Rachel Carson

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INTRODUÇÃO

Comportamento reprodutivo em insetos

A reprodução sexuada é o modo mais comum de reprodução entre os insetos (Gillott 2005, Gullan & Cranston 2012), e inclui todos os eventos que envolvem a inseminação (transferência dos espermatozoides) até a fertilização (fusão entre os gametas feminino e masculino) (Gillott 2005, Matthews & Matthews 2010). Entretanto, a fertilização poderá ocorrer logo após a cópula (ex. em *Drosophila*) ou muito tempo depois (ex. em formigas e abelhas) (Bloch *et al.* 2003, Collins *et al.* 2004), sendo assim a inseminação e fertilização nos insetos são dois eventos dissociados determinantes do sistema de acasalamento e reprodução da espécie (Matthews & Matthews 2010).

A realização da cópula e subsequente inseminação da fêmea dependem de vários comportamentos pré-cópula (i.e., de corte), durante e pós-cópula (Gillott 2005, Gullan & Cranston 2012). Assim, a reprodução e os eventos envolvidos, tornam-se um processo vital para qualquer espécie e, em especial, para aquelas de reprodução sexuada, com chance de selecionar um parceiro sexual. Contudo, sabe-se que alguns grupos/espécies de insetos podem se reproduzir assexuadamente tais como pulgões, cochonilhas, mosca-branca, parasitoides entre outros (Gillott 2005, Matthews & Matthews 2010, Gullan & Cranston 2012, Olzer *et al.* 2018). Isto ocorre porque a meiose, processo pelo qual se originam os gametas, é drasticamente modificada, fazendo com que o gameta resultante seja diploide e não necessite ser fertilizado para se desenvolver, resultando em reprodução por partenogênese (Gilbert 2003). Também, dependendo das condições ambientais bióticas (entomopatógenos, simbiontes, tamanho da população e disponibilidade de recurso) e abióticas (temperatura e fotoperíodo) algumas espécies de insetos podem muitas vezes ter gerações alternadas que se reproduzem de forma assexuada e sexuada (Goudie & Oldroyd 2014, Leonardo & Mondor 2006, Simon *et al.* 2010).

Os sistemas de acasalamento observados em insetos incluem: monogamia, quando macho e fêmea copulam apenas um com o outro durante um ciclo reprodutivo ou toda a vida; poligamia, quando um dos pares possui mais de um parceiro durante o período reprodutivo; e promiscuidade, quando ambos são poligâmicos. Quando a poligamia é expressada apenas por um dos sexos, esta recebe nome de poliginia quando é expressada pelos machos, e poliandria quando é expressada pelas fêmeas (Matthews & Matthews 2010, Olzer *et al.* 2018). Apesar dos custos atrelados à poligamia, este é o comportamento apresentado pela maioria dos insetos (Gullan & Cranston 2012).

Em algumas espécies, a cópula em si é capaz de reduzir a sobrevivência dos machos, como relatado para *Adalia bipunctata* (L.) (Coleoptera: Coccinellidae), e múltiplas cópulas diminuem a capacidade destes de transferir espermatóforos em futuros acasalamentos (Perry & Tse 2013). Em outros grupos de insetos, como nos em percevejos Cimicidae, múltiplas cópulas resultam em injurias e aquisição de patógenos, afetando a capacidade reprodutiva e a longevidade das fêmeas (Reinhardt *et al*. 2005). Isto ocorre porque neste grupo a fecundação se dá exclusivamente por inseminação traumática, com o macho inserindo a genitália diretamente na cavidade abdominal da fêmea (Stutt & Siva-Jothy 2001). Por outro lado, o comportamento poligâmico também oferece benefícios para uma população, não só em termos de variabilidade genética (Michaud *et al.* 2013), como também no aumento da fertilidade e no desenvolvimento da descendência, sobretudo em fêmeas poliândricas como foi observado na joaninha *Hippodamia convergens* Guérin-Méneville (Coleoptera: Coccinellidae) (Bayoumy & Michaud 2014). Por fim, há espécies em que a poligamia não leva a um ganho reprodutivo aparente, como na joaninha *Tenuisvalvae notata*

(Mulsant), que apesar da fêmea copular com mais de um macho em um curto período de tempo (< 24h), não existe um aumento significativo no desempenho da mesma (Túler *et al.* 2018).

Múltiplas cópulas, combinado a outros fatores como fecundação interna, armazenamento de espermatozoides e sobreposição de esperma de diferentes machos podem resultar em competição espermática (Gullan & Cranston 2012). Ainda, a aquisição de material espermático de diferentes machos pode intensificar a competição espermática, favorecendo os espermatozoides com melhor aptidão média (Bayoumy & Michaud 2014). Na maioria das vezes, a precedência do esperma é do último macho (De Jong *et al.* 1993, Ueno 1996, Chaudhary, Mishra & Omkar 2016). Isto pode ocorrer devido a fatores como pouca capacidade de armazenamento da espermateca, deslocamento do material armazenado pelo esperma recém adquirido (Ueno 1996), ou devido à ejeção e utilização de espermatóforo como alimento, liberando espaço para o esperma de outro macho (Obata & Hidaka 1987). Em Odonata, os machos possuem adaptações na morfologia dos seus órgãos reprodutivos e no comportamento de acasalamento, que permitem remover o esperma de outro, além de guardar a fêmea após copular até a oviposição (Snodgrass 1935, Corbet 1980). Outras espécies inoculam aleloquímicos nas fêmeas, inibindo sua receptividade para outros machos (Gillott 2003, Matthews & Matthews 2010, Avila *et al.* 2011, Olzer *et al.* 2018).

Comportamento reprodutivo em joaninhas

As joaninhas são predominantemente poligâmicas, isto porque as fêmeas da maioria das espécies necessitam de várias cópulas para manter uma alta fertilidade (Arnqvist & Nilsson 2000, Omkar & Singh 2010, Omkar *et al.* 2010). A cópula em joaninhas é semelhante à dos demais coleópteros, com o macho subindo nos élitros da fêmea e sendo carregado por ela (Nedved & Honek 2012, Santos *et al.* 2017). Entretanto, de maneira geral, antes da cópula propriamente dita há uma série de rituais ou comportamentos de corte que envolvem procura, reconhecimento e aceitação, até que se realize a monta, cópula e posterior separação do casal (Matthews & Matthews 2010). A corte é uma etapa fundamental para a reprodução em coccinelídeos e as ações executadas pelos machos durante a corte não visam impressionar a fêmea, como ocorre em outros grupos de insetos, isto devido à falta de elaboração desta etapa neste grupo (Omkar & Singh 2010). Em algumas espécies (i.e., *Coccinella septempunctata* Linnaeus e *Coelophora saucia* (Mulsant), a corte consiste basicamente da abordagem, observação, exame (antenação), montagem e tentativa de cópula (Omkar & Srivastava 2002, Omkar & Singh 2010). Tais ações, possivelmente, tem o intuito de apaziguar a fêmea a fim de evitar a rejeição, além de se certificar do sexo (macho ou fêmea) e da espécie do parceiro (Gillott 2005, Omkar & Singh 2010). A execução de tais comportamentos irá depender de características intrínsecas da espécie. Contudo, na joaninha objeto deste estudo, *E. connexa*, o comportamento de corte não foi relatado (D'Ávila *et al.* 2018a) e, ainda, há espécies onde a fêmea é o sexo ativo que tenta levar o macho para à cópula, como em *T. notata* (Santos *et al.* 2017).

Para a maioria das espécies, existe um período para a maturação sexual, e o acasalamento é influenciado pelo fotoperíodo e outras características físicas como temperatura e umidade. Além disso, fatores como a quantidade e qualidade do alimento ingerido, densidade populacional e número de cópulas, também podem interferir na maturação sexual de uma espécie (Gillott 2003, Santos *et al.* 2017). O período de maturação sexual é bastante variável, podendo ocorrer em apenas uma hora após a emergência, como em fêmeas de *T. notata* (Santos *et al.* 2017). No entanto, há espécies que necessitam de vários dias para tornarem-se maduras sexualmente, como ocorre em *Harmonia axyridis* (Pallas) (Obata 1988). Além disso, a maturidade sexual também pode diferir entre os sexos, sendo chamada protandria, quando os machos se tornam maduros primeiro, e protoginia, quando o mesmo ocorre com as fêmeas (Obata 1987, Nedved & Honek

2012). Em *Cheilomenes sexmaculata* (Fabr.), os machos estão maduros sexualmente aos dois dias de vida adulta, enquanto as fêmeas em cerca de quatro dias (Bind 2007). Em *T. notata*, os machos estão maduros sexualmente com quatro dias de idade, enquanto as fêmeas estão aptas para acasalar em menos de 24 horas após a emergência (Santos *et al.* 2017). Em *Cryptolaemus montrouzieri* Mulsant, as fêmeas demoram, em média, três semanas para completar o desenvolvimento dos ovaríolos. Porém nos machos, apesar da maturação dos testículos ocorrer em cerca de uma semana, só há transferência espermatozoides em 20 a 30 dias após a emergência, tempo necessário para que os músculos de bloqueio do ducto ejaculatório se tornem totalmente funcionais. Portanto, sem a ação destes músculos nenhum espermatozoide pode ser transferido e os machos não realizam cópulas (Kaufmann 1996), indicando que a maturidade sexual pode ser um processo multifatorial, não dependendo unicamente da maturação das gônadas.

A aceitação de um parceiro sexual geralmente se dá pela fêmea. Assim, a fêmea pode aceitar ou não um determinado macho para copular, o que parece estar intimamente relacionado com a maturidade sexual da fêmea (Obata 1988). Contudo, em *T. notata*, diferente do que ocorre na maioria das espécies, é a fêmea que corteja o macho. Nesta espécie, a duração média da cópula é apenas 84 segundos, mas para isso acontecer muitas vezes a fêmea passa horas cortejando o macho, até que ele aceite copular (Santos *et al.* 2017). Em contrapartida, há espécies que apresentam cópulas mais demoradas. Em *C. septempunctata* L., a duração da cópula é de 54 minutos, em média (Rana & Kakker 2000). Em *H. axyridis*, a cópula dura de duas a três horas (Obata 1987), e há registro de casos extremos, como o de *Aiolocaria hexaspilota* (Hope), em que os indivíduos permanecem em posição copulatória durante vários dias (Iwata 1932). Além da variação de duração, o repertório comportamental também é bastante variável entre as espécies. Em *H. axyridis* os machos agitam o corpo repetidas vezes durante a cópula, para transferir o material espermático para a bursa copulatrix da fêmea (Obata 1987). Tal comportamento de agitação, no entanto, não é observado em *H. convergens* (Bayoumy & Michaud 2014) e em *E. connexa* (D'Ávila *et al.* 2018a).

Após a cópula, o material seminal pode ser utilizado imediatamente para fertilizar os óvulos já desenvolvidos no oviduto, ou pode ser armazenado por dias ou até meses, em estruturas especiais denominadas espermateca (Chapman 1998). Os espermatozoides são transferidos envoltos em um espermatóforo, ou podem ser liberados diretamente na espermateca (Chapman 1998, Gillott 2003). Quando existe armazenamento de material seminal, este pode ser originário de apenas uma cópula (monoandria), ou de múltiplas cópulas com vários parceiros (poliandria), podendo a fêmea, em algumas situações, selecionar quais espermatozoides usar (De Jong *et al.* 1993). Um exemplo é o observado em fêmeas de *H. axyridis*, as quais costumam ejetar e utilizar o espermatóforo como recurso nutricional extra (Obata & Hidaka 1987). Este comportamento acaba favorecendo o material do macho seguinte, reafirmando a questão da precedência do esperma que, na maioria das vezes, é do último macho a acasalar (De Jong *et al.* 1993, Ueno 1996). Por outro lado, há espécies em que ocorre a mistura de material seminal na espermateca e nesse caso a fêmea não pode selecionar qual material seminal usar primeiro. Por exemplo, em *C. montrouzieri*, uma fêmea pode ter seus ovos fertilizados pelos espermatozoides de até quatro machos diferentes (Kaufmann 1996, Kairo *et al.* 2013).

Em Coccinellidae, questões como a anfimixia e a poligamia são bem definidas, contudo estudos recentes registraram a ocorrência de reprodução por partenogênese telítoca, em duas populações selvagens de *Nephus voeltzkowi* Weise e partenogênese arrenótoca em uma população de laboratório de *C. montrouzieri*, ambas coccidófagas (Magro *et al.* 2020, El Aalaoui *et al.* 2021). No primeiro caso, é possível que a origem da partenogênese esteja relacionada à interação com a bactéria *Wolbachia pipientis* Hertig. Isto porque nas populações estudadas foram encontrados apenas indivíduos do sexo feminino, e a infecção por *Wolbachia* foi confirmada em todos os descendentes dessas populações. Contudo, esta hipótese não foi confirmada, uma vez que não foi possível determinar se a infecção ocorreu antes ou após a transição da anfimixia para a partenogênese (Magro *et al.* 2020). Além disso, os autores mencionam que indução infecciosa da partenogênese obrigatória só é confirmada em organismos haplodiploides, e não em diplodiploides como em joaninhas (Magro *et al.* 2020). No segundo exemplo, os autores sugerem que a partenogênese poderia ter sido induzida pelos componentes das ceras cuticulares presentes na cochonilha *Dactylopius opuntiae* (Cockerell), utilizada como presa pela espécie, uma vez que não ocorreu reprodução partenogenética em indivíduos alimentados com a presa cujas ceras cuticulares foram removidas, porém os mecanismos desta interação não foram elucidados (El Aalaoui *et al.* 2021).

Comportamento de oviposição em joaninhas

Em coccinelídeos, ovos e larvas recém-eclodidas representam os estágios de maior vulnerabilidade devido à baixa mobilidade e a fraca resistência do tegumento (Polis 1981). Imediatamente após a eclosão, as larvas de coccinelídeos permancem sobre os ovos, alimentando-se do fluido remanescente de seus próprios ovos, até completarem a esclerotização do tegumento (Brown 1972). Antes de se dispersarem, as larvas permanecem agrupadas próximo à postura por pelo menos um dia, consumindo ovos não fertilizados (Banks 1956, Osawa 1989). Em larvas jovens, é frequente a ocorrência de canibalismo de ovos da mesma postura (“sibling-cannibalism”), ou de posturas não-relacionadas (“non-sibling”) (Osawa 1989, Honek & Evans 2012). A frequência desses comportamentos varia em função da densidade da presa no ambiente e, geralmente, ocorrem com indivíduos maiores ou em estágios de desenvolvimento mais avançados predando os menores ou de estágios iniciais (Polis 1981, Polis, Mayers & Holt 1989, Oliveira 2020).

Apesar disso, diferentemente de outros grupos de insetos, comportamentos de cuidado parental não são observados em joaninhas (Honêk 1996). Contudo, a escolha da fêmea pelo local de oviposição sugere algum nível de proteção dos ovos depositados e parece estar relacionada ao comportamento dos imaturos após eclosão. Assim, as fêmeas podem ter evoluído para selecionar locais de oviposição que minimizem a predação (Griffin & Yeargan 2002, Staley & Yeargan 2005). Como estratégia de provisão, fêmeas de algumas espécies costumam depositar seus ovos em locais próximos à uma fonte de alimento, como colônias de pulgões (Honêk 1996). Contudo, esse comportamento também pode resultar em canibalismo ou predação intraguilda, uma vez que nesses locais há uma alta frequência de encontros de larvas e adultos com as posturas depositadas, fato que algumas espécies ovipositam a uma certa distância das presas (Honek & Evans 2012). Esta dualidade é vista como um “trade-off” entre o potencial risco de escassez para os descendentes, caso os ovos sejam depositados muito distantes da fonte de alimento, e sua potencial perda por canibalismo ou predação intraguilda, caso sejam depositados muito próximos (Michaud & Jyoti 2007).

Portanto, para reduzir a frequência de encontros entre indivíduos forrageadores com as posturas ou larvas recém eclodidas, as fêmeas buscam locais que dificultem esses encontros (Lucas 2012). Fêmeas de *Coleomegilla maculata* (DeGeer), por exemplo, selecionam plantas com tricomas glandulares para depositar seus ovos, independente da densidade de presa nessas plantas (Griffin & Yeargan 2002, Staley & Yeargan 2005), provavelmente devido à ocorrência de alta predação de ovos, especialmente por canibalismo, em plantas com pouca ou nenhuma pubescência (Staley & Yeargan 2005). Estudos com *Serangium japonicum* Chapin sugerem que esta preferência ocorre devido à arquitetura da superfície abaxial foliar, na qual a densidade dos tricomas diminui a taxa de encontro de predadores com as posturas (Yao *et al.* 2021). Além disso, a preferência de oviposição nessa espécie também parece estar relacionada com a fixação do inseto na planta, uma vez que os tricomas conferem uma maior interação com os tarsos dos adultos (Yao *et al.* 2021). Em laboratório, fêmeas de *C. maculata* foram observadas ovipositando na face interna das sépalas de botões florais de algodoeiro (*Gossypium hirustum* L.) e nas dobras de folhas enroladas de *Commelina comunis* L., enquanto em *E. connexa*, as posturas são frequentemente coletadas em folhas de papel toalha amassadas e na superfície abaxial de folhas de couve (*Brassica oleracea* L.) infestadas com pulgões (*obs. pessoal*).

A sinalização química também tem sido relacionada com a proteção das posturas em Coccinellidae. Fêmeas inseminadas utilizam sinais químicos, deixados por indivíduos conspecíficos ou não, a fim de evitar a oviposição em locais com indícios da presença de potenciais canibais ou predadores intraguilda, conforme observado em *Cheilomenes sexmaculata* (Fabr.) (Ruzicka 2006). Em *Hippodamia convergens* Guérin-Méneville, as fêmeas também evitaram ovipositar em substratos contaminados com resíduos de *C. maculata*, e mais ainda quando se tratava de resíduos de conspecíficos (Michaud & Jyoti 2007). Além disso, a sinalização química também tem função de alertar o predador sobre possíveis riscos. Aliás, a própria superfície dos ovos é impregnada com secreções contendo compostos de defesa ou de sinalização, produzidas pelas glândulas acessórias da fêmea, conforme observado em *A. bipunctata* e *C. septempunctata* (Hemptinne *et al.* 2000, Katsanis *et al.* 2017).

Ademais, fêmeas de Coccinellidae podem depositar ovos não fertilizados para alimentar os descendentes, sendo este um comportamento adaptativo materno, dependente da disponibilidade de recursos, ou seja, as fêmeas produzem mais ovos inférteis em condições de escassez do que em abundância de presas (Polis 1981, Perry & Roitberg 2005). Ovos não fertilizados e ovos contendo embriões não eclodidos são comuns em posturas de Coccinellidae, e ambos são consumidos pelas larvas, que se beneficiam desse comportamento, principalmente pela chance de sobreviver até encontrar sua primeira presa (Brown 1972). Os ovos constituem em alimento de grande valor nutricional devido ao seu alto teor lipídico e proteico, além de outros componentes em menor quantidade, como glicogênio e carboidratos (Chapman 1998, Slogett & Lorenz 2008). Além disso, estudos já demonstraram que a ingestão de um único ovo aumentou significativamente as chances de sobrevivência de larvas neonatas de pelo menos quatro espécies de joaninhas (Elnagdy *et al.* 2011). Em *Coccinella undecimpunctata* L., o canibalismo de ovos por indivíduos adultos conferiu benefícios para a fecundidade e fertilidade das fêmeas, além de reduzir o período de desenvolvimento dos descendentes, principalmente quando ambos os pais eram canibais (Bayoumy *et al.* 2016). Polis (1981) menciona que ovos não eclodidos são presas fáceis para as joaninhas e a ingestão desses ovos melhora sua chance de sobrevivência pela aquisição de nutrientes. O canibalismo de ovos é comum em criações de laboratório de *E. connexa* (Silva *et al.* 2010). Para contornar a situação da criação de vários indivíduos juntos, são ofertados presa em abundância e papel amassado para aumentar a superfície de oviposição, a fim de mitigar este comportamento como descrito na metodologia de Rodrigues *et al.* (2013a, 2014). Além disso, estudos demonstram que a frequência do canibalismo de ovos em *E. connexa* é maior na ausência de presas (Rocca *et al.* 2020), corroborando com o que se observa em outros coccinelídeos. Contudo, questões acerca dos efeitos do canibalismo de ovos no desenvolvimento e no desempenho reprodutivo de *E. connexa*, ainda, não foram estudados.

Aplicabilidade do estudo

Estudos relacionados com o comportamento de acasalamento em Coccinellidae podem auxiliar no entendimento de aspectos evolutivos (Arnqvist & Nilsson 2000), de aspectos práticos como definir protocolos de criações, e em estudos para identificação de semioquímicos envolvidos na atratividade do parceiro sexual, visando a manipulação de populações, em campo (Santos 2016), e na própria caracterização comportamental da espécie (Santos *et al.* 2017). A idade ideal de acasalamento, o número de cópulas necessárias e os intervalos entre elas podem mostrar uma correlação de cópulas com o desempenho (*fitness*) da espécie, através de incremento na fecundidade, fertilidade e longevidade das fêmeas (Mishra 2005, Haddrill *et al.* 2007, Omkar *et al.* 2010, Colares *et al.* 2015). Estudos para extração de glândulas ou das substâncias químicas envolvidas na cópula, exigem o conhecimento da idade e do período ideal do dia em que a espécie expressa os comportamentos de acasalamento (Aldrich *et al.* 1999, Gillott 2003).

Apesar da potencialidade da aplicação do conhecimento a ser gerado pela manipulação comportamental de joaninhas para aumento do uso no controle biológico, bem como da criação em laboratório, existem poucos estudos quanto ao entendimento do comportamento reprodutivo de *E. connexa*. Mesmo que os comportamentos de corte possam ser estereotipados, a escolha do parceiro, idade de cópula, uso do material seminal e outros comportamentos podem ser espécie-específicos, tais como a fêmea utiliza os espermatozoides e coordena a produção de ovos férteis. Assim, questões ainda precisam ser elucidadas como aquela relacionada a precedência espermática, ou porque *E. connexa* mantém o comportamento poliândrico, quando este não oferece ganhos na fecundidade ou viabilidade (Colares *et al.* 2015). Para responder a essas e outras questões, estudos de comportamento reprodutivo têm se apropriado de diversas metodologias, porém o entendimento da precedência de espermatozoides é difícil, por se tratar da separação dos descendentes de diferentes machos de uma mesma espécie, obtidos de subsequentes ejaculações.

Nesse contexto, uma opção para elucidar a paternidade em Coccinellidae é a utilização de marcadores moleculares. Ao utilizar machos transportando diferentes marcadores genéticos, a descendência pode ser genotipada a fim de definir qual macho é o pai (Sloggett & Honěk 2012). Esta metodologia foi utilizada para investigar a precedência espermática em *H. axyridis* e, apesar de serem observados descendentes de até 10 machos diferentes em fêmeas de campo, verificou-se que a maior porcentagem de ovos foi fertilizada por quatro machos (Awad *et al.* 2015). Além disso, acasalamentos sequenciais demonstraram que, em 60% das fêmeas analisadas, a maioria dos descendentes foi do primeiro macho a acasalar (Nedvěd *et al.* 2018).

A esterilização por radiações ionizantes pode também ser utilizada para investigar a paternidade em insetos. Estudos demonstraram que a precedência do esperma em fêmeas de *Rhynchophorus ferrugineus* (Olivier) (Coleoptera: Curculionidae) foi do último macho acasalado. Nesses casos, a precedência é determinada pela viabilidade dos ovos de fêmeas após acasalamentos consecutivos (Musmeci *et al.* 2018). Apesar da praticidade, além da esterilização, a irradiação pode ocasionar efeitos indesejados no comportamento reprodutivo, como foi observado em *Anastrepha serpentina* (Wied.) (Diptera: Tephritidae), na qual machos irradiados apresentaram menor média de cópulas e da capacidade de induzir a refratariedade nas fêmeas (Landeta-Escamilla *et al.* 2016).

Outra metodologia, ainda não explorada, é a determinação da paternidade através da herança da resistência a inseticidas. Parentais resistentes transmitem esta característica aos descendentes, por meio de um determinado fator genético pré-existente. A resistência constitui-se em uma característica genética, hereditária e pré-adaptativa (Oppenoorth & Welling 1976). Estudos com *E. connexa*, identificaram a resistência à lambda-cialotrina mediada por enzimas destoxificativas (Rodrigues *et al.* 2014) e transmitida de forma autossômica (Rodrigues *et al.* 2013a), sem alterações morfológicas ou comportamentais que possam impossibilitar a reprodução (Spíndola *et al.* 2013, D'Ávila *et al.* 2018a). Assim, tal característica pode ser utilizada para testar a paternidade, uma vez que descendentes de ambos os sexos herdam a resistência. Dessa forma, quando os descendentes são expostos à dose letal do inseticida para o indivíduo suscetível, estes são distinguidos dos resistentes e a paternidade determinada pela taxa de sobrevivência dos descendentes.

Além da resistência passada aos descendentes dando a esses maior probabilidade de sobreviverem às aplicações de inseticidas nos agroecossistemas, a progênie também precisa ter presa suficiente para completar seu desenvolvimento. A fase larval, em especial larvas recém-eclodidas de predadores são sensíveis a falta de alimento por apresentarem baixa dispersão, serem usualmente menores que as presas disponíveis, principalmente para larvas neonatas de Coccinellidae (Banks 1956, Osawa 1989). Em *E. connexa*, tem sido observado que fêmeas fazem posturas com ovos inférteis em meio a ovos férteis, mesmo quando recentemente acasaladas, quando teoricamente estariam com estoque suficiente de espermatozoides para fertilizar todos os seus ovos (Colares *et al.* 2015). A produção de ovos é de elevado custo para a fêmea, e teoricamente todos os ovos deveriam ser convertidos em novos indivíduos (Bateman 1948, Trivers 1972, Chapman 1998, Slogett & Lorenz 2008).

Um dos principais efeitos observados quando insetos adultos são submetidos a escassez de alimento é a redução da fecundidade como sendo um "trade-off" energético em favor da sobrevivência (Resende *et al.* 2015, Santos *et al.* 2016, Chaudhary *et al.* 2018, Lima, Pontes & Nóbrega 2020, Nascimento *et al.* 2021). Assim, a deposição de ovos inférteis é considerada como uma estratégia de investimento na descendência pela disponibilização de alimento para larvas recém-eclodidas, visto que essas se alimentam de ovos coespecíficos logo após a eclosão (Polis 1981, Perry & Roitberg 2005, 2006). A utilização de ovos inférteis, às vezes denominados de ovos tróficos, também é comum em insetos como himenópteros (Koedam *et al.* 1996, Fang & Mueller 2019), percevejos subsociais (Hironaka *et al.* 2005, Baba *et al.* 2011, Kudo *et al.* 2021), e em alguns coleópteros como passalídeos (Ento *et al.* 2008) e crisomelídeos (Tigreros *et al.* 2017).

Em fêmeas de *E. connexa*, mesmo em condições de pareamento contínuo com machos, ocorre a deposição de ovos inférteis (Colares *et al.* 2015, Lira *et al.* 2016, Nascimento *et al.* 2021). Assim, neste trabalho de tese, também realizamos estudos para entender tal comportamento e se a produção de ovos inférteis está associada a fatores extrínsecos, como a disponibilidade de presas no ambiente, conforme demonstrado para outras espécies (Polis 1981, Perry & Roitberg 2005). Além disso, interações como canibalismo e predação intraguilda podem interferir na dinâmica das populações de inimigos naturais, comprometendo o resultado esperado (Vilela & Pallini 2002). Assim nossos resultados poderão contribuir para o conhecimento das interações entre os indivíduos de uma mesma população de *E. connexa*, e desses indivíduos com outros membros da guilda trófica, contribuindo para a conservação desta espécie.

Relevância e objetivos do estudo

Os coccinelídeos compreendem mais de seis mil espécies descritas em todo o mundo (Vandenberg 2002) e, com exceção dos membros da subfamília Epilachninae, exclusivamente fitófaga, as demais espécies são fungívoras ou predadoras, sendo este último grupo o mais representativo (Giorgi *et al.* 2009, Castro-Guedes 2013). As joaninhas predadoras são agentes de controle biológico de artrópodes de corpo mole como pulgões, cochonilhas, psilídeos, ácaros, larvas de pequenos lepidópteros, entre outros (Hodek & Honêk 1996, Obrycki & Kring 1998, Biddinger *et al.* 2009, Prasad *et al.* 2009, Schuber *et al.* 2012).

Várias espécies de joaninhas são utilizadas em programas de controle biológico aplicado ou conservativo, em várias partes do mundo, sendo ainda consideradas como predador modelo para a divulgação do controle biológico. O controle biológico aplicado com joaninhas foi o marco inicial para o controle biológico clássico (Hajek & Eilenberg 2018). Entre os diversos exemplos, podemos citar o caso de *Cryptolaemus montrouzieri* Mulsant, introduzida na Califórnia, EUA, para o controle de cochonilhas em citros (Kairo *et al.* 2013), e também no Brasil, em 1988 (Sanches & Carvalho 2011); e que continua sendo introduzida em outras regiões para o controle de cochonilhas (Qin *et al.* 2019). Já no controle biológico conservativo, temos a espécie *E. connexa*, a qual é nativa da América do Sul (González 2014) e, no Brasil, é frequentemente encontrada predando afídeos em plantas ornamentais, frutíferas e hortaliças (Sujii *et al.* 2007, Harterreiten-Souza *et al*. 2012, Schuber *et al.* 2012, Rodrigues *et al.* 2013b, Dalazen *et al.* 2017, Costa *et al.* 2018, Fidelis 2018, Soares *et al*. 2020). Embora seja principalmente afidófaga, *E. connexa* também é capaz de predar ovos de lepidópteros, ácaros, psilídeos e larvas de pequenos lepidópteros (Sarmento *et al.* 2007, Silva *et al.* 2013b, Lira *et al.* 2019, Nascimento *et al.* 2021).

Devido ao seu papel no controle biológico, estudos sobre *E. connexa* tem investigado seu comportamento alimentar (Sarmento *et al.* 2007, Tulli, Carmona & Vincini 2013, Lira *et al.* 2019), a utilização de presa alternativa em criações (Silva *et al.* 2013a, Santos *et al.* 2016), a biologia da espécie (Miller & Paustian 1992, Miller 1995, Silva *et al.* 2013b), técnicas de criação utilizando dieta artificial (Martos *et al.* 1992, Silva *et al.* 2009); resposta a inseticidas (Fogel *et al.* 2013, Fogel *et al.* 2016, Rimoldi *et al.* 2017, Bestete *et al.* 2018, D'Ávila *et al.* 2018b, Pasini *et al.* 2018, Costa *et al.* 2020), suscetibilidade a inseticidas (Gusmão *et al.* 2000, Venzon *et al.* 2007, Tavares *et al.* 2010, Machado *et al.* 2019), e caracterização e herança da resistência a inseticidas piretroides (Rodrigues *et al.* 2013a, Rodrigues *et al.* 2014, Costa *et al.* 2018).

Diante do potencial de *E. connexa* para o controle biológico percebe-se a necessidade de ampliar os estudos acerca da espécie, a fim de maximizar sua utilização em programas de Manejo Integrado de Pragas. Apesar da importância, ainda há questões a serem respondidas acerca do sucesso reprodutivo de *E. connexa,* como a precedência de esperma e fatores que influenciam na fecundidade e na fertilidade desta espécie, como a escassez de presa, canibalismo e predação intraguilda. Assim, este estudo irá contribuir com a divulgação de dados inéditos sobre estas questões para *E. connexa*. Para tal, foram levantadas as hipóteses de que *E. connexa* necessita de um período para alcançar a maturação sexual, apresentando atividade de cópula durante a fotofase, uma vez que coccinelídeos são insetos diurnos (i); *E. connexa* é uma espécie promíscua, não havendo precedência espermática, em vez disso, ocorre mistura de espermatozoides após acasalamentos sequenciais (ii), o que pode ser estudado empregando o fenótipo de resistência a inseticidas, permitindo determinar a paternidade da descendência; e por último, se joaninhas depositam ovos não fertilizados a fim de prover uma primeira refeição para as larvas neonatas, então a produção de ovos inférteis por *E. connexa* pode ser maximizada em situações de experiência de escassez de presas (iii). Sendo assim, este estudo buscou esclarecer aspectos acerca do comportamento sexual e reprodutivo de *E. connexa*, como o início da maturidade sexual, o período de maior atividade sexual na fotofase e a paternidade dos descendentes; conhecer a influência do estresse alimentar e do canibalismo no desenvolvimento e no desempenho reprodutivo desta espécie, bem como elucidar a função da manutenção deste comportamento em condições de abundância de presas.

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CAPÍTULO 2

SEXUAL MATURITY, PARTNER CHOICE AND SPERM PRECEDENCE IN THE PROMISCUOUS LADYBIRD BEETLE *Eriopis connexa* (GERMAR): WHO IS MY FATHER?[1]

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[1] Silva, A.C.G. Sexual maturity, partner choice and sperm precedence in the promiscuous ladybird beetle *Eriopis connexa* (Germar): who is my father? Behavioural Processes 192, 104500, 2021 (common names and scientific terms cited according to the journal guidelines).

MATURIDADE SEXUAL, ESCOLHA DO PARECEIRO E PRECEDÊNCIA ESPERMÁTICA NA JOANINHA PREDADORA, *Eriopis connexa* (GERMAR): QUEM É MEU PAI?

RESUMO **-** Joaninhas constituem predadores-chave de muitas pragas agrícolas. Dentre elas, *Eriopis connexa* se destaca por apresentar algumas características importantes para programas de manejo de pragas como: ampla distribuição, população selecionada para resistência a inseticidas piretroides e possibilidade de criação com presa alternativa. Apesar disso, pouco se sabe sobre o papel da poliandria e seu efeito na paternidade dessa espécie. As joaninhas se envolvem em múltiplos acasalamentos, levantando questões sobre os benefícios da poliandria e a paternidade. Nós estudamos a seleção do parceiro para o acasalamento, a precedência dos espermatozoides e a paternidade dos descendentes realizando experimentos sobre (1) a idade de maturidade sexual, (2) o comportamento refratário e a frequência de acasalamento durante a fotofase, (3) a preferência de fêmeas suscetíveis para acasalar com machos suscetíveis ou resistentes; e (4) o efeito de múltiplos acasalamentos na paternidade da progênie. A maturidade sexual em *E. connexa* tem início aos 3 dias após emergência do adulto em ambos os sexos, mas só está totalmente desenvolvida após 5 dias. A maior frequência de acasalamento exibida por pares sexualmente maduros ocorreu na primeira hora da fotofase e o fenótipo para resistência não influenciou a escolha do parceiro. O fenótipo resistente, usado como marcador, indica que a paternidade da progênie resulta da mistura de espermatozoides disponíveis e não depende da ordem de acasalamento. Esses dados sugerem que joaninhas liberadas teriam uma perspectiva simples para a produção de progênie tanto para joaninhas suscetíveis quanto resistentes a inseticidas.

PALAVRAS-CHAVE: Reprodução, maturidade sexual, seleção sexual, fenótipo de resistência.

ABSTRACT - Ladybird beetles present as key predator of many agricultural pests. Among them, *Eriopis connexa* stands out due some important traits for pest management programs such as common occurrence, population selected for resistance to pyrethroid insecticides, and possibility to be commercially available by rearing using alternative prey. Despite that, little is known about the role of polyandry and its effect on paternity in this species. Ladybird beetles engage in multiple matings, raising questions about the benefits of polyandry and paternity. We studied the selection of mating pair, sperm precedence, and offspring paternity by performing experiments on (1) the age of sexual maturity, (2) the frequency and refractory mating behaviour within a photophase period, (3) the preference of insecticide-susceptible females to mate with either susceptible or resistant males; and (4) the effect of multiple matings on progeny paternity. Sexual maturity in *E. connexa* was found to begin within 3 days of adult emergence for both sexes, but fully developed after 5 days. The highest frequency of mating exhibited by sexual mature pairs occurred within first hour of pairing and the phenotype for insecticide resistance did not affect the choice of a mating partner. The resistance trait marker in the resistant beetle phenotype indicates that progeny paternity results from a mixture of available sperm and do not depend on mating order. These data suggest that released beetles would have a simple prospect for progeny production for both insecticide resistant and susceptible beetles.

KEY WORDS: Reproduction, sexual maturity, sexual selection, resistance phenotype.

Introduction

Early offspring production results in shorter generation times and faster population growth, and thus this trait should be subjected to positive selection in habitats with variable resources. In ladybird beetles, the age of sexual maturation is highly variable (Richards 1980, Obata 1988, Hemptinne *et al.* 2001, Santos *et al.* 2017), and males and females may reach sexual maturation at different ages, causing protogyny, in which the maturation of the female sex organs precedes that of the males, and protandry, in which, on the contrary, the male sex organs mature before the females (Nedvěd & Honek 2012). Females of the predatory ladybird beetle *Tenuisvalvae notata* Mulsant become receptive to mating a few hours after adult emergence, while males require about four days. Therefore, *T. notata* females are responsible for the courtship repertoire to induce males to mate (Santos *et al.* 2017). On the other hand, females of *Cryptolaemus montrouzieri* Mulsant require about 10 days to be receptive to mating, and males take 10 to 15 days to reach sexual maturation **(**Kairo *et al.* 2013). For the ladybird beetle *Eriopis connexa* (Germar), there is no well-defined age for sexual maturity, but it has been considered to be five days or more after emergence (Mirande *et al.* 2010, Colares *et al.* 2015, D'Ávila *et al.* 2018). Males and females of this species do not have any external morphological sexual dimorphism, but female at this age exhibit dilated abdomen due to oogenesis and are highly receptive to mating, but the timing of male sexual maturity is less clear.

*S*imilarly, to many other ladybird beetle species, *E. connexa* mates with different males throughout the female's life, even within a single day (Colares *et al.* 2015). Overall, polygamy in coccinellids leads to enhanced fecundity and fertility (Arnqvist & Nilsson 2000; Srivastava & Omkar 2005, Omkar & Singh 2010). In *E. connexa*, however, multiple matings with either virgin or mated males does not affect female fecundity (Colares *et al.* 2015). Therefore, studies on this aspect are important to optimize insectary rearing after knowing the best conditions to reproduce the species, and to make ecological and evolutionary inferences about the species regarding the parental participation on the offspring performance transferring traits that enhances their survival in the crop ecosystem.

Regarding the reproductive behaviour of coccinellids, identification of paternity is difficult because of the potential for sperm competition in species with promiscuous females. For instance, studies using molecular analyzes confirmed the occurrence of sperm precedence (with dominance in fertilization in this case by the first mate) in *H. axyridis* (Awad *et al.* 2017, Nedvěd *et al.* 2018). In *Menochilus sexmaculatus* (Fabr.) fertilization outcomes following sequential matings support sperm precedence of the last male; however, if the second mating occurs after some interval or when the first mating was of greater duration, the paternity percentages due to each male become similar, indicating sperm mixing within the female's spermatheca (Chaudhary *et al.* 2016, Dubey *et al.* 2018).

As mentioned, *E. connexa* lacks sexual dimorphism becoming hard to set apart newly molted adults males and females. Furthermore, this species exhibit analogous-mating repertoire between pyrethroid-resistant and susceptible populations (D'Ávila *et al.* 2018). The resistant trait can be used to discriminate susceptible and resistant individuals by counting the survivors after a treatment with the pyrethroid insecticide. The pyrethroid-resistant individuals differentiate from susceptible ones by enhanced enzymatic detoxification (Rodrigues *et al.* 2014), but not morfologically. This increased metabolic function in the resistant phenotype has reproductive costs that include delaying in egg-laying and lower female fecundity, although both resistant and susceptible females show similar longevity. Both can live on average 60 days, but some individuals may live longer (Ferreira *et al.* 2013, Lira *et al.* 2016, Rodrigues *et al.* 2016). However, no information is available on reproductive costs (i.e., sperm viability) in resistant males, irrespective of the phenotype. Furthermore, the resistance phenotype is inherited as an autosomal trait in *E. connexa* (Rodrigues *et al.* 2013, Lira *et al.* 2016), what will allow us to determine the paternity of the progeny and the degree of occurrence of sperm precedence in this ladybird beetle species. We can separate the phenotypes by treating them with a dose of the pyrethroid lambda-cyhalothrin that kills the susceptible individuals in the progeny. Thus, to investigate the paternity, first we had to clarify some mating behaviour regarding the age of males and females reach the sexual maturity (1), the period of the greatest mating activity (2), and the duration of mating (3) to ascertain about subsequent mating refractoriness. Then, we could investigate the effect of sperm precedence and progeny paternity in *E. connexa considering* the offspring phenotype as resistant (Res), susceptible (Sus) or a mixture of both types, to see how progeny type depended on the phenotype of the male used in either the first or second mating with a susceptible female. We hypothesize that even if not fully mature males can mate with mature females, these females would experience early sperm depletion (1). In some cases, females may select males with specific traits for mating because mating with superior males (the good genes hypothesis) fitter offspring. However, we hypothesize that resistant trait exhibited by *E. connexa* does not influence mate selection because of lacking an external sign (i.e., morfological clue) (2), and because of promiscuos female behaviour in this species there is sperm mix in the spermatheca (3), resulting in a lack of precedence for sperm from either the first or last mating male.

Material and Methods

We ran five experiments. First, we defined the ages of sexual maturity for males and females with a series of pairings beginning with one-day-old adults. Second, we investigated the hour of highest mating frequency within a 12h-photophase period. Third, we determined if the resistant trait exhibited by *E. connexa* had an influence on partner choice. Finally, given that we found that females showed no preference for mating with resistant versus susceptible males, we investigated the link between multiple matings in *E. connexa* and the resulting sperm competition on the observed paternity of the resulting offspring. All experiments were conducted at 25 ± 1 °C, photoperiod 12:12 (L:D), and 60 to 70% r.h. in the “Laboratório de Controle Biológico” of the “Universidade Federal Rural de Pernambuco (UFRPE)”, Recife, PE, Brazil.

The two ladybird beetle populations: susceptible (Sus) and resistant (Res) (hereafter resistant and susceptible phenotypes) were obtained from field-collected insects and have been maintained under the same physical conditions and food availability in the laboratory since their field collections in 2008 and 2009, respectively (Torres *et al.* 2020, Rodrigues *et al.* 2020). The pyrethroid-susceptible phenotype was collected from cotton fields in Frei Miguelinho County (Pernambuco State, Brazil) with annual renew with insects collected during the cotton crop season, and they are reared without insecticide exposure. The resistant phenotype was originally collected from cabbage fields (Rodrigues *et al.* 2013), and implemented with resistant individuals from other locations after resistance testing (Costa *et al*. 2018), which has been periodically treated with lambda-cyhalothrin at technical grade and checked for resistance in previous studies (Rodrigues *et al.* 2020).

Rearing of *Eriopis connexa.* The rearing procedures followed Rodrigues *et al.* (2013), with the modification that adults were maintained in transparent Plexiglas™ cages (40 × 25 × 20 cm of L × H × W) with lateral openings closed with organdie fabric for ventilation. These cages were considered as the free-choice mating arenas and adult rearing cages. By using these large cages, more than 100 males and females were able to mate and produce eggs. Larvae and adults were fed ad libitum with eggs of *Ephestia kuehniella* (Zeller) (Lep.: Pyralidae). Adult diet was also supplemented with a past of honey: yeast (1:1), plus aphids or psyllids when available.

Using insects from the susceptible phenotype, we determined age of sexual maturation, the duration of mating, and the periodicity of mating across a 12h-photophase. Pupae of *E. connexa* were monitored twice each day to obtain new adults on their day of adult eclosion. After eclosion, new adults were reared individually to produce males and females of known age to use in assays.

Assay 1: Estimating Age of First Mating. The number of days required for Sus males and females to reach sexual maturity was determined through two tests. First, we defined the onset of mating receptivity. Adults of both sexes were held in isolation until they were the desired age to be tested. Because young males and females cannot be distinguished without dissection, for 1- to 4-day old test groups we used the free-choice mating in the Plexiglas rearing cage. Individuals that were either 1 or 2 days old, were placed in groups of 100 in the same cages as used for rearing of adult beetles, and observed continuously from 8:00 h until 17:00 h for occurrence (or not) of mating. Neither 1- or 2 days-old adults mated or tried to mate. Similarly, in the second test, adults of 3- and 4-day old were placed in free-choice mating cage in groups of 85 and 79 individuals for age 3 and 4-days, respectively. Couples initiating mating were gingerly transferred to Petri dishes (9 × 1.5 cm in diam × H) to avoid the copula disengage, and, after mating ended, males were discarded but females were held for 10 days and daily fecundity and fertility recorded. For older adults (5 to 10 days old) females can be identified by their dilated abdomens. For these age groups, beetles were placed in Petri dishes in pairs. Due to the different emergence dates in these groups, the number of pairs per age varied, ranging from 14 to 18. Oviposition was used to indicate that a mating had been successful (Santos *et al.*, 2017).

Assay 2: Male Maturity and Sperm Production. From 5- to 6-day-old, isolated beetles, females were selected based on the enlarged abdomens. These Sus females were then paired with Sus males that were 3, 4, or 5 days old to determine when males are first sexually mature. We defined "sexually mature" males when they were able to fertilize females indirectly measured through egg fertility. A mating event does not mean sexual maturity because males can mate with non-sexually mature females resulting in a lack of offspring production immediately (Hemptinne *et al.*, 2001; Nedvěd and Honek, 2012). Thus, we assumed that males mating could occur before total maturity. Younger males (1 or 2 days of age) were not capable to mate as previously noted, and thus they were not used in this assay. Thus, three treatments were set up: 3-day-old males (n = 13 couples), 4-day-old males (n = 12 couples), and 5-day-old males (n = 12 couples). Couples were allowed to mate and, later, when mating ended, males were discarded, and females were monitored for oviposition for 30 days. During observation period, we recorded female survival, fecundity, and the fertility (egg hatching rate), with this last variable being used as an indirect measure of male sexual maturity.

Assay 3: Frequency of Mating. We run this assay to determine the mating frequency after pairing sexually mature females and males in the photophase. Furthermore, it was possible to evaluate the refractory behaviour of females after successfully completing a mating that could interfere with the second order male mating during the paternity assay. Thus, 29 pairs of virgin sexually mature males and females (10 days old) were isolated in Petri dishes (9 × 1.5 cm in diam × H), and pairs were continuously observed from 7:00h until 19:00h under laboratory conditions as described in the rearing section. By direct observation, we recorded the time to first mating, mating duration, and the number of matings for each pair within 12h-photophase observation period. These data were collected during two successive days of observations to have males and females of the same age and run the observations.

Assay 4: Mating Preference between Insecticide Susceptible vs. Resistant Phenotypes. To test the hypothesis that females in our system could make such a discrimination, we allowed susceptible females to choose between susceptible (♂Sus) and resistant (♂Res) males for mating. The hypothesis was tested using two different experiment design: (1) simultaneous free-choice between Res and Sus males to test a possible female discrimination between Sus or Res males, and (2) sequential mating by females between Res and then Sus males, to test for possible refractory behaviour right after the first mating. Refractoriness can represent a mechanism to favor sperm fertilization of the first male. In each test, 82 males and 41 females of each genotype were paired as further described for each test. The final number (replications) analyzed on each test was 40 females that successfully accomplished both mating and, therefore considered each as a replication.

In the first test (simultaneous free choice), one virgin 5-day-old female was released into a Petri dish (9 × 1.5 cm high) containing one Res and one Sus virgin male of the same age. The female was continuously observed to record the female’s first partner choice. After the onset of mating, the rejected male (Sus or Res) was transferred to another dish and later used to provide the female with a second mating opportunity. Right after ending the first mating, the female was transferred to the dish containing the previously rejected virgin male. Thus, the mated female was allowed to mate with the male rejected during the first choice.

In the second test (sequential mating), two pairings were carried out sequentially to determine if there was any preference for the first partner choice by the female. Susceptible females were used as they are readily available in nature. Thus, the female was allowed to have a first mate under non-choice condition either with one Sus (i) or Res (ii) male. Resistant males received a small red mark with nontoxic ink on the pronotum with the help of an entomological pin to differentiate then from Sus males. Previous tests showed that this procedure did not affect female’s mating choice. Right after the female had completed the first mating, the second virgin male of the opposite phenotype was introduced into the dish together the first male, under choice condition. Thus, the female was subjected to choose between the first mated male and one virgin male of opposite phenotype. At beginning of the second mate, the rejected male was discarded, and the mating couple followed to the end of mating. Data measured included (1) the phenotype of the chosen male, (2) elapsed times to first and second matings, and (3) the duration of the first and second matings.

Assay 5: Sperm Precedence and Paternity. After determining the age of sexual maturation of both males and females, the highest frequency of mating within the first hour of the photophase using receptive and mature pairs, and the lack of female preference between susceptible vs. resistant male phenotypes, and refractory time for second mating, then we could run this study to investigate the sperm precedence. We run the assay testing the progeny produced by females subjected to two successive matings with either a Res or Sus male phenotype. It is known that lambda-cyhalothrin resistance inheritance in *E. connexa* is dominant and autosomal, hence, from crossing a Res male with a Sus female or vice-versa, the F1 progeny would be the resistant phenotype (Rodrigues *et al.* 2013, Lira *et al.* 2016). Thus, the resistant phenotype was used as a marker trait to assess paternity to progeny of females mated with males either Res or Sus. We used sexual mature 8-day-old virgin females of both phenotypes (susceptible [♀Sus] or resistant [♀Res]). These females were paired with a resistant (♂Res) or susceptible (♂Sus) male. Four pairing combinations were setup: (1) ♀Sus×♂(Sus<Sus) (control for expected progeny 100% Sus); (2) ♀Sus×♂(Res<Sus); (3) ♀Sus×♂(Sus<Res); and (4) ♀Res×♂(Res<Res) (control for expected progeny 100% Res). The number of insects that completed the sequential pairings resulted in 12 replications for pairings (2) and (3), and 10 females each for pairings (1) and (4). Females were confined in Petri dishes (9 × 1.5 cm) and had one first male available for mating according to the planned pairing. Couples were continuously observed to record the beginning and the ending of each mating bout. Right after the first mating, the female was offered a second male for mating according to respective treatment. Again, the couple was observed in order to record the times when any second mating began and ended. After finishing the second mating, females were transferred to 80 mL

transparent pots with pieces of paper towel as an oviposition substrate and an abundance of *A. kuehniella* eggs as food. Egg production was checked daily for each female and pairing. After eclosion, larvae were reared 3-4 days and then subjected to insecticide treatment to check for resistance, in which larvae were topically treated with 1 μL of a previously determined dose of 1.0 mg a.i./mL of lambda-cyhalothrin at technical grade (99.5%; Chem Service, West Chester, PA, EUA) diluted into acetone, using a 25 μL Hamilton™ syringe (Hamilton Company, Reno, NV, USA) attached to a controlled repeat applicator. The exposure to this dose was expected to cause 100% and 0% mortality for larvae of the Sus and Res phenotype, respectively. After treatment, the larvae were transferred to new Petri dishes (9 × 1.5 high) lined with filter paper and fed *A. kuehniella* eggs. Larval mortality was assessed 48 h after treatment. The criterion for mortality was an individual's inability to turn itself upright and begin to walk, after being placed on its back. This procedure was repeated with larvae from 1st, 3rd, 5th, 7th, 9th, 11th and 13rd egg batches produced after the pairings, being 48 to 55 larvae per pairing treatment and date, depending on the available number of larvae. Thus, respectively for pairings ♀Sus×♂(Sus<Sus), ♀Res×♂(Res×Res), ♀Sus×♂(Sus<Res), and ♀Sus×♂(Res×Sus), 341, 367, 353, and 373 larvae were treated to generate the outcome.

Data Analysis. For Assay 1 on age of first mating, fecundity and fertility from each pairing were square root (x + 0.5) and log (x +1) transformed, respectively, to fit the assumptions of the analysis of variance (ANOVA). Further, one-way ANOVA was performed comparing ages as treatments for males and females separately. To run the comparisons regarding the different ages (n = 8), we took the Sidăk-Bonferroni's correction approach (0.05/n; $\alpha = 0.00625$) to keep alpha error equal 0.05. The differences in fecundity and fertility were separated by Tukey HSD test at 0.05 significance levels (SAS Institute 2002).

For Assay 2, male maturity and sperm production, the data were submitted to one-way ANOVA with ages of males as treatments, and Tukey HSD's tests were performed for mean separation, with a 0.05 significance levels (SAS Institute 2002).

For Assay 3, on the time of photophase when mating was most frequent, data for all parameters were tested for equal frequency among hours of the photophase through the Proc PROC FREQ of SAS (SAS Institute 2002). Means and 95% confidence intervals of the time required to the first mating, duration of mating, and mating refractoriness (time to engage the next mating) per couple were calculated through the PROC MEANS of SAS (SAS Institute 2002).

For Assay 4 (female preference between susceptible and pyrethroid-resistant males), female mating choice as a function of the male phenotypes Res or Sus was submitted to the Chi-square test ($\alpha = 0.05$) through the PROC FREQ of SAS (SAS Institute 2002) to test the hypothesis of similarity between phenotypes (50: 50). Because data on mating duration did not fit the ANOVA assumptions despite data transformation, the data were analyzed using nonparametric analysis with PROC NPAR1WAY of SAS (SAS Institute 2002) and comparisons performed with the Wilcoxon's test.

For Assay 5 (sperm precedence), data from fecundity during the 30-day observation period for Sus and Res females paired with Res or Sus males were square root $(x + 0.5)$ transformed to fit the ANOVA assumptions. The data were then subjected to one-way ANOVA and Tukey HSD test ($\alpha = 0.05$) for mean comparisons. The sperm precedence and offspring paternity outcome according to the female and male phenotypes pairings was tested through the offspring mortality when the larvae were treated with the pyrethroid lambda- cyhalothrin. The PROC FREQ of SAS (SAS Institute 2002) and Chi-square's tests were performed to compare observed and expected mortalities based on the hypothesis of sperm competition as follows: 100% offspring mortality expected from pairing ♀Sus×♂(Sus<Sus), 50% offspring mortality expected

from pairings ♀Sus×♂(Res<Sus) and ♀Sus×♂(Sus<Res); and 0% offspring mortality expected from pairing ♀Res×♂(Res<Res).

Results

Assay 1: Estimating Age of First Mating. Both males and females of *E. connexa* exhibited mating receptivity at similar age and obtained fertilization when 3 days old. Despite that, age of the beetle couple did affect female fecundity (F = 8.38, df = 7, 632, P < 0.0001) and the fertility (F = 5.37, df = 7, 463, P < 0.0001), both of which were higher at 5- and 6-days of age for the couple, compared to couples that were 3- or 4-days old (Fig. 1).

Assay 2: Male Maturity and Sperm Production. In the previous assay, 5-day-old females that were mated to males of the same age showed higher fecundity and fertility rates compared to younger (3 or 4 days old) pairs of beetles. However, since both males and females' ages changed between treatments, the lower performance of young couples may have been due to the age of one but not necessarily both sexes. Thus, in this Assay 2, we paired sexually mature, 5- or 6-day-old females with 3-, 4-, or 5- day-old males to ascertain about the maximum ability of males to fertilize females. Based on that, we will have certainty about the age of male to be paired for testing male paternity. The data resulted in higher rates of fertilization (egg hatching) when mature females of a standard age were paired with older males (F = 3.67, df = 2, 28, P = 0.03) (Fig. 2A). Females that mated with 5-day-old males produced eggs with higher fertility (68.7 ± 4.1%) compared to females that mated with 3-day-old males (40.1 ± 8.5%), while results were intermediate for females mated to 4-day-old males (47.8 ± 9.0%) (Fig. 2A). Male age did not affect total monthly female fecundity, with egg production averaging from 347.4 ± 74.4 to 440.5 ± 26.2 eggs per female over 30 days (F = 0.77, df = 2, 28, P = 0.47). Fertility of females mated once with 5-day-old males was greater than 50% during the whole 30 days observation period, with values up to 80%; in contrast, females mated to younger males exhibited sperm depletion such that by the end of the 30 day observation period female fertility was down to 20 to 30% (Fig. 2B).

Assay 3: Frequency of Mating. Sexual maturity couples exhibited the highest frequency of mating during the first hour of pairing ($\chi^2 = 49.51$, df = 11, $P < 0.0001$) (Fig. 3). During this period, 100% of pairs mated at least once, with four pairs mating more than once. The average time until the first mating (latency time) and the refractory time (mean time between successive matings) were 13.7 ± 1.6 and 60 ± 5.8 min, respectively. The average mating duration was 23.9 ± 1.78 min, but the value varied from 5 to 90 minutes. The mean number of matings accomplished by females was 6.1 ± 0.8 during the 12h photophase observation period. The number of matings was also highly variable, with three couples (of 29 couples) mating only once, whereas one couple (of 29 couples) mated 21 times in a 12h-photophase period.

Assay 4: Mating Preference between Insecticides Susceptible vs. Resistant Phenotypes. No female mate preference was found between resistant and susceptible male phenotypes ($\chi^2 = 0.45$, df = 1, $P = 0.5011$). Likewise, when offered a previously known male and a new male, females did not show any mate preference ($\chi^2 = 1.67$, df = 1, $P = 0.19$). For second matings, refractoriness (time between matings) was not different between Res and Sus males ($\chi^2 = 0.89$, df = 1, $P = 0.34$). Similarly, mating duration did not differ with Res versus Sus males ($\chi^2 = 0.23$, df = 1, $P = 0.63$). Finally, the average refractory time ($\chi^2 = 1.58$, df = 1; $P = 0.21$) and mating duration ($\chi^2 = 0.08$, df = 1, $P = 0.77$) of the second mating did not differ statistically between Sus and Res males.

Again, when females were subjected to sequential matings, there was no difference among the series of matings for refractoriness for either Sus ($\chi^2 = 0.15$, df = 1, $P = 0.69$) or Res males ($\chi^2 = 1.74$, df = 1, $P = 0.19$). Likewise, there was no difference among the series of matings in mating duration with either Sus ($\chi^2 = 0.006$, df = 1, $P = 0.9385$) or Res males ($\chi^2 = 1.33$, df = 1, $P = 0.25$).

3.5. Assay 5: Sperm Precedence and Paternity. Knowing that *E. connexa* female does not exhibit preference for male pair between resistant (Res) and susceptible (Sus) phenotypes neither exhibited refractoriness in sequential matings, this experiment could be done. Females × male × male pairings in all four tested combinations – ♀Sus×♂(Sus<Sus), ♀Sus×♂(Res<Sus), ♀Sus×♂(Sus<Res); and ♀Res×♂(Res<Res) – where the first entry is the female's phenotype, all females produced similar numbers of eggs per batch for all 13 batches produced in the experiment ($F_{3, 40}$ = 0.10, P = 0.958), with an average varying from 312.0 to 343.3 eggs per female (225.3 and 456.9 eggs per female [95% CI]). When the resultant larvae were tested for possible inherited trait regarding the resistance to lambda-cyhalothrin as a marker (larvae from 1st, 3rd, 5th, 7th, 9th, 11th and 13th egg batches) showed that the proportion of fertilized eggs of each progeny fitted the expected larval mortality associated with the known male and female parental phenotypes (Fig. 4 shows only the 1st and 13th egg batches and the overall average). Furthermore, the overall estimates across all tested larvae fit the sperm competition hypothesis (χ^2 = 4.93, P = 0.18) (See definition in M&M section). The offspring produced was a mixture of Res and Sus phenotypes, irrespective of the female's first partner choice and age of egg batch produced.

Discussion

Knowledge of insect reproduction increases our understanding of topics useful for manipulation of pest populations and for better appreciation of insect evolution (Matthews & Matthews 2010). Detailed knowledge of insect reproductive behaviour helps improve rearing techniques needed to provide material for various purposes and studies, and allows us to formulate and test hypotheses about the evolution. Topics such as partner choice for mating and how females gain benefits through mate selection or employing a strategy of polyandry and sperm competition to maximize the quality and the number of offspring produced can be addressed.

Most female and male insects need a period after emergence to reach sexual maturity (Obata 1988, Nedvěd & Honek 2012, Santos *et al.* 2017). For the ladybird beetle *E. connexa* both sexes were not receptive to mate in the first two days of adult age requiring time to attain maximum reproductive success. Often, insect species defer mating if one of the pair is not sexually mature. However, in some species, sexually mature males may coerce females to mate before they are sexually mature (Roth & Willis 1955, Seeley & Dukas 2011, Olzer *et al.* 2018), and in such cases, there may be no immediate offspring production. Therefore, a mating event does not necessarily guarantee reproductive success. Mating coercion is common in species where females only mate once, triggering greater competition among males (Matthews & Matthews 2010, Olzer *et al.* 2018). In the ladybird beetle *Propylea dissecta* (Mulsant), females were observed mating with sexually mature males after being coerced (Omkar & Pervez 2005). In the ladybird beetle *Leptothea galbula* (Mulsant), males guard female pupae to guarantee a mate opportunity immediately after female emergence (Richards 1980). This type of mating, before females reach sexual maturity, does not result in immediate fertilization of eggs, but because females can storage the seminal material in the spermatheca, it enhances a female's fecundity and fertility when she attains sexually mature (Hemptinne *et al.* 2001, Nedvěd & Honek 2012).

In *E. connexa,* some mating occurred in 3-day-old adults, with some able to lay viable eggs, despite the fact that full sexual maturity did not occur until day five. This result suggests that in our test species mating receptivity develops earlier than full sexual maturation, for both sexes. In our test species, the first four days of adult life may have been required for maturation of the reproductive organs of both sexes, since early mating resulting in both low fecundity (indicative of inadequacies by the female) and a low fertility (indicative of low sperm transference from males). These results suggest that *E. connexa* exhibits gradual sexual maturation between the 3rd and the 5-6th day after emergence at which time both fecundity and fertility stabilize at maximum levels. Similar results were found in another ladybird beetle, *Harmonia axyridis* (Pallas), in which oocyte production began when adult females were 3 days old, but reached its maximum when females were 7 days old (Obata 1988).

For males of *E. connexa*, gradual sexual maturation significantly affected the number or the quality of sperm transferred to the females, resulting in a fewer progeny being produced from mating with males that were less than 3 to 5 days old. Females of *E. connexa* that mated with males that were mature (5 days old or older) had higher offspring production for up to 30 days post mating, while fewer than 50% of the eggs laid by females that mated with young males (3-4 days old) hatched. Interestingly, female fertility never reached 100% even when females mated with mature males. Thus, we may ask why females do not fertilize all the eggs laid in the first egg batch? We are aware that there is a cost of producing oocytes and the fact that females of *E. connexa* acquire enough spermatozoa from a single mating to fertilize eggs for up to 30 days. Several hypotheses have been advanced to explain this phenomenon, one that seems to fit our system concerns possible nutritional deficits in newly hatched larvae (Osawa, 1992). When such deficits exist, females may lay unfertilized eggs (a.k.a. trophic eggs) so that newly hatched larvae can eat such eggs and avoid cannibalism among siblings (Perry & Roitberg 2006). Indeed, Perry & Roitberg (2005) found that food deficiencies led to an increase in the number of unviable eggs being laid by *H. axyridis*. We noticed that newly eclosed larvae of *E. connexa* remained for several hours aggregated over their natal egg batch, and if larvae were isolated right after eclosion, they experienced a higher rate mortality.

In *E. connexa*, couples mated several times in the course of one 12h-photophase, following 10 days in the adult age without mating. Copulation was initiated in less than one minute being allowed access to the opposite sex, and mating lasted for about 24 min, corroborating the results from Colares *et al.* (2015). The observation of a great number of matings per couple (up to 21 times) in a short period, as in our test species after lacking a mating, may be due to the experimental setup with continuous pairing favoring the contact between sexes. In *Propylea dissecta* (Mulsant), a reduction on mating duration was observed as the number of matings increased (Omkar & Pervez 2005). In *Podisus nigrispinus* (Dallas) (Hemiptera: Pentatomidae), for which a high number of matings was noted, reduction of mating duration was observed for subsequent matings with reduction of transference of seminal material (Rodrigues *et al.* 2008). In the ladybird beetle *Menochilus sexmaculatus* (Fabr.), long-term matings did not result in increased sperm transference, but a post-copulation guarding behaviour (Chaudhary *et al.* 2015). Likewise, in *E. connexa*, the duration of mating did not affect the fertility (D'Ávila *et al.* 2018). In some species, such as *H. axyridis*, the transfer of spermatozoa only takes place when the male exhibits the particular behaviour of "body shaking". Mating stopped before this period not result in female fertilization (Obata 1987). Males of *E. connexa* did not engage in this behaviour in our study. Females of *E. connexa* flick their bodies as a type of behaviour, but this behaviour is not related to sperm transfer or mating duration. The female body flicking was observed right before copulation started after the male mounting the female's body, and when a female was ending a mating (D'Ávila *et al.* 2018) and appeared to help females get rid of the male.

Regarding the mating frequency, sexual maturity couples initiated mating right after the pairings. Despite sexual maturity, light plays significant role in ladybird beetle mating. The ladybird beetle *T. notata* did not exhibit any sexual or foraging activities during the scotophase (Santos *et al.* 2017). Ladybird beetles exhibit diurnal activities such as foraging for food and mating (Nakamuta 1984, 1987, Santos *et al.* 2017). *In situ* field studies have recorded *E. connexa* visiting flowers during the night but less frequently than during the daytime (Torretta *et al.* 2009, 2010). However, the presence of a ladybird beetle on flowers at night does not necessarily indicate nocturnal feeding since the flower might only be being used as a shelter.

In *E. connexa*, mating frequency for sex mature couples was greatest during the first hour of pairing (100% of couples mated) with a continuous decrease of mating frequency as the day progressed (Fig. 3). It seemed likely to decrease mating frequency along the observation period as the female became fertilized and the male had mated one or more times. Ladybird beetles are diurnal, their sexual activity may be influenced by the environmental lighting conditions and the circadian rhythm of each species (Nakamuta 1984, 1987, Omkar *et al.* 2005, Omkar & Pathak 2006). It is worth mentioning that our data were obtained with sexually mature beetles and paired after first hour of pairing. Thus, the higher frequency of matings within the first hours after pairing may be occurred due to combination of these two factors.

Females of *E. connexa* did not show any mating preference between susceptible and resistant virgin males, either when offered simultaneously or in sequence. The lack of preference is essential information for using the resistant phenotype in applied biological control. It is expected that wild susceptible individuals mate with released resistant phenotype to produce a resistant F1 offspring (Lira *et al.* 2016, Rodrigues *et al.* 2020) due to the dominant and autosomal mode of inheritance of the resistance to lambda-cyhalothrin in *E. connexa* (Rodrigues *et al.* 2013). Experiments aiming to test partner choice by females should consider the role of male competition and try to mitigate its effects (Kivleniece *et al.* 2010). Even so, we found no evidence of these effects for *E. connexa*. Therefore, the lack of preference between resistant or susceptible phenotypes becomes an advantage for the species' conservation in crop ecosystems that apply pyrethroid insecticides (Torres & Bueno 2018).

Promiscuity, a form of polygamy with random mating among insect adults as exhibited by *E. connexa*, has been the subject of extensive research (Arnqvist & Nilsson 2000, Simmons 2005, Jayanthi *et al.* 2013, Bayoumy & Michaud 2014, Awad *et al.* 2015, Colares *et al.* 2015, Bista 2019) with interesting evolutionary behavioural outcomes. In polyandrous reproduction systems, there is a coexistence of sperm from different males, leading to sperm competition for egg fertilization (Parker 1970, 1984, Simmons 2005). Evolutionarily, polyandry has been considered of great benefit for genetic diversity and offspring vigor (Arnqvist & Nilsson 2000, Simmons 2005), although some drawbacks may exist regarding transference of pathogens to the females, female stress and risks of injury during mating. Overall, sperm precedence is common in polyandrous females, “the last in, the first out” hypothesis (De Jong *et al.* 1993, Chaudhary *et al.* 2016). However, our results indicate that offspring produced are from different fathers in similar proportions regardless of mating order.

Sperm mixing acts as an adaptive mechanism of intrasexual selection, and several processes are involved, leading to the superior performance of one male sperm over the rival male sperm (Parker 1970). One adaptation in polygamous species is the production of superior sperm (viability, mobility, faster swim rate, etc.) compared to sperm from monogamous species (Hunter & Birkhead 2002). Because evolution favors the superior sperm, sperm competition is mediated by several different genes, including those involved in sperm development, morphology, viability, and motility (Civetta & Ranz 2019).

In insects, the spermatozoa may be freely transferred to females (direct transference) or packed in spermatophores, which are transferred to the females (indirect transference) (Chapman 1998, Fassotte *et al.* 2016). In *E. connexa*, there is no previous information regarding sperm transference, and, based on our observations, sperm transference is direct through female insemination. Also, in our study species there was no behaviour indicative of spermatophore transfer, as was observed in *H. axyridis* (male body shaking) (Obata 1987, Colares *et al.* 2015, D’Ávila *et al.* 2018). Furthermore, behaviours related to pre-mating selection (Santos *et al.* 2017) and post-mating behaviours such as female guarding (Alcock 1994), ejection and ingestion of seminal material (Obata & Hidaka 1987) were not observed also in *E. connexa* (Colares *et al.*

2015, D'Ávila *et al.* 2018). These findings strongly suggest that in *E. connexa* sperm mixing drives post-mating factor of sexual selection.

Therefore, in the present study we found that sexual activity in *E. connexa* starts on day 3 but that beetles only reach full sexual maturity, in both sexes, 5 days after emergence. Also, sexual activities take place during the first hours of pairing with decreasing mating frequency as the day progresses. Females of *E. connexa* do not exhibit any preference for mating with mated or virgin males and when they are phenotypically different regarding susceptibility or resistance to pyrethroids. Polyandrous mating behaviour in *E. connexa* females does not select for sperm precedence, but for an equated use of sperm within the spermatheca, with similar chances of offspring paternity between males regardless of the mating order indicating the absence of sperm precedence in *E. connexa* as a function of male phenotypes. Further knowledge about the mechanism of female fertilization in *E. connexa* such as the presence or absence of a spermatophore, the reasons for female promiscuity in the face of no gains in fecundity or fertility, and other variables will help us better understand the mating behaviour in this species. Our findings contribute to our understanding of reproduction, mating the lack of influences of the adult phenotype, and determinants of offspring paternity for this important biological control agent, especially in regards the application of the resistant phenotype in biological control programs. The release of the Res individuals and their subsequent cross with field susceptible individuals will not be impaired by the phenotype carrying the resistant trait, and the crossing with either males or females will result in about 50% offspring resistant at the first generation.

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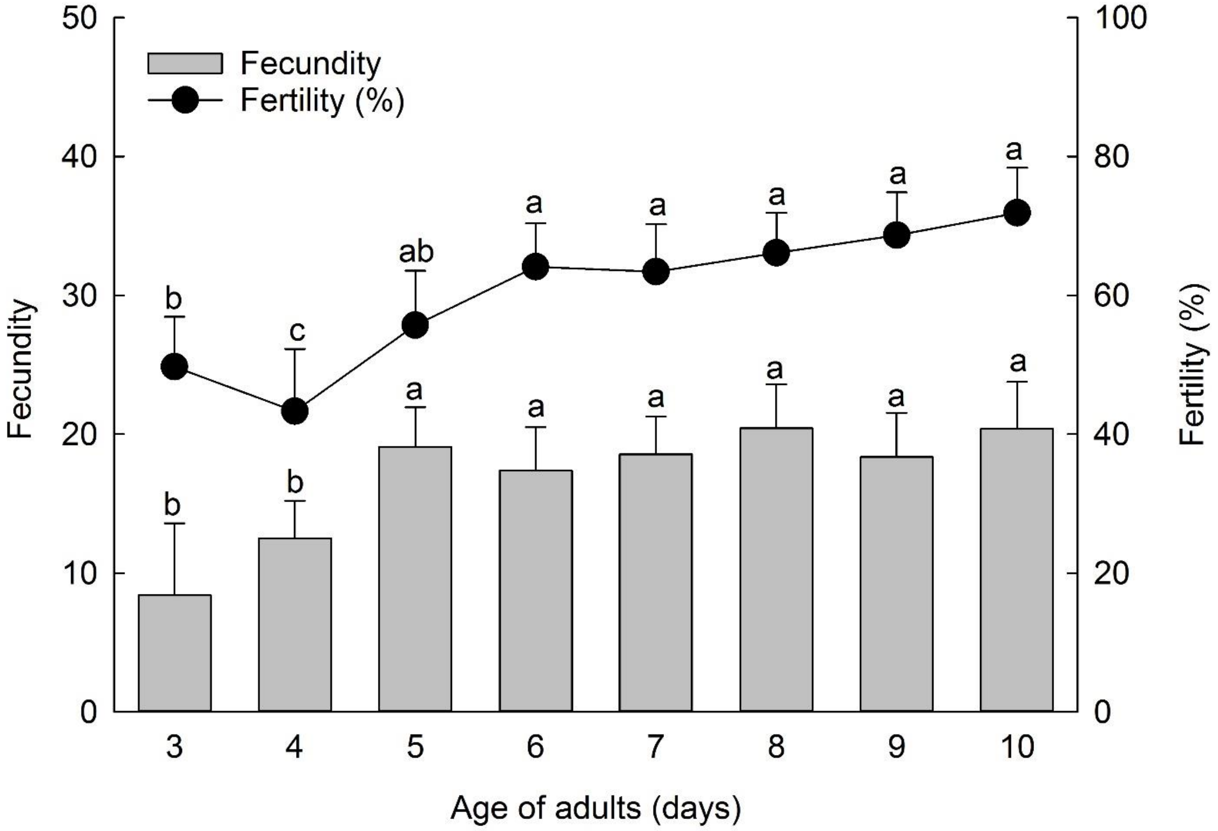

Figure 1. Fecundity as eggs per female and fertility (% egg hatching) for *Eriopis connexa* couples for the first 10 days after initiation of mating on day 3 after adult eclosion. Means (+SE) are different by Tukey HSD's test (α = 0.00625) when followed by different letters.

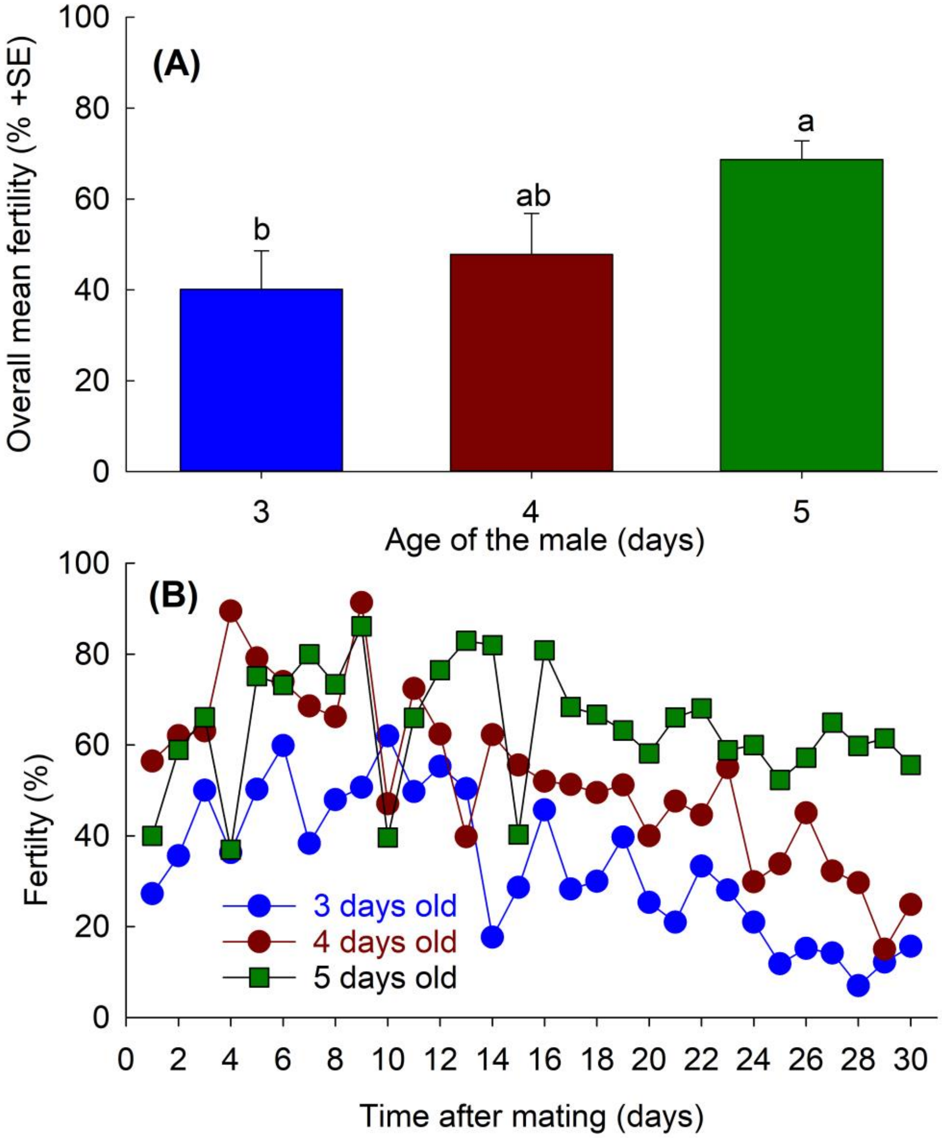

Figure 2. Effect of male age and sperm depletion. **A**: Fertility (mean +SE for egg hatching) when females of standard age (5 or 6 days) were mated with males from 3 to 5 days of age; **B**: fertility over a 30-day period, showing sperm depletion rate in relation to age of males mated to test females. Different letters indicate significant difference by Tukey HSD' test (α = 0.05).

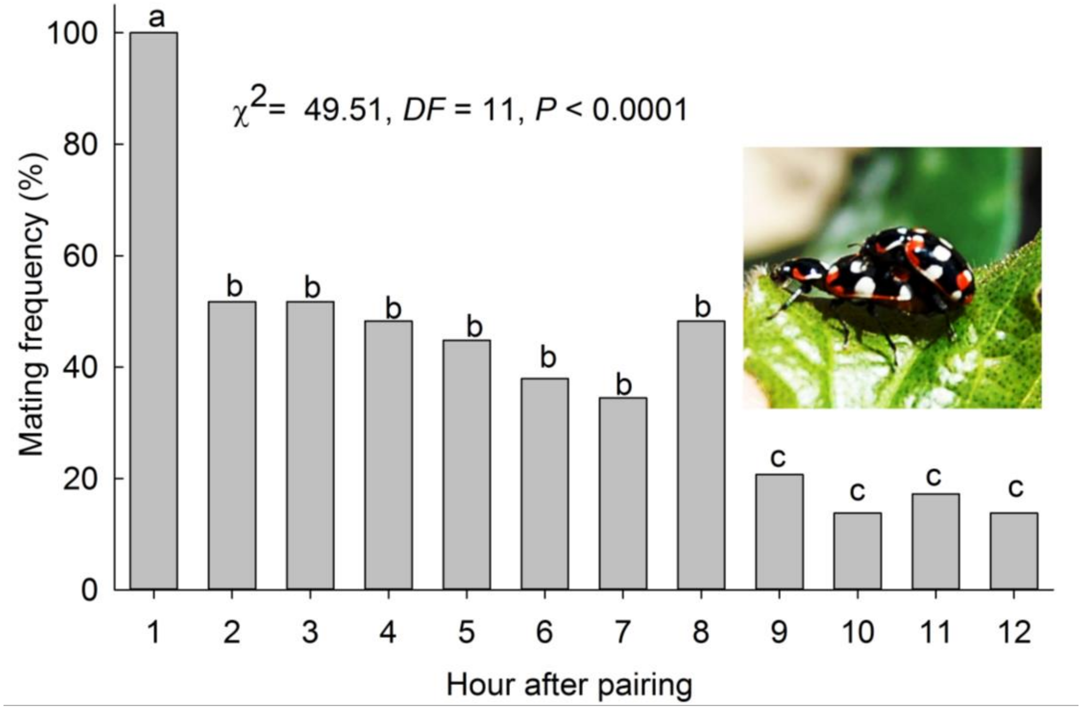

Figure 3. Mating frequency for sexual maturity *Eriopis connexa* pairs in the photophase period (7:00h to 19:00h). Different letters indicate difference in mating frequency found by Chi- square tests comparing each hour to the previous hour within the observation period (α = 0.05).

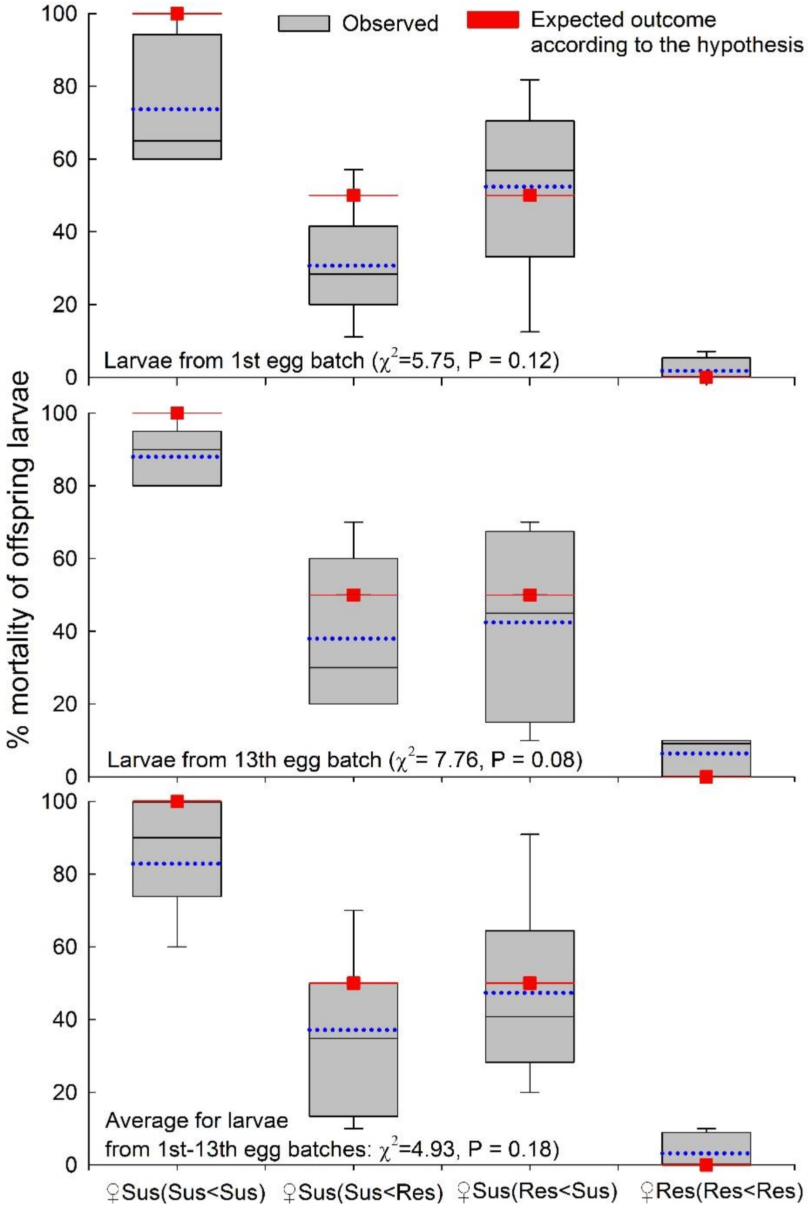

Figure 4. Mortality of *Eriopis connexa* larvae (here showed for 1st, 13th and the overall average) treated with lambda-cyhalothrin to identify the source of offspring paternity, distinguishing susceptible (Sus) versus resistant (Res) males. Acronyms between parentheses stand for male running the first and second matings. Continuous lines and dotted lines denote the median and mean values of observed mortality, while red square symbols indicate the expected mortality outcome for the offspring according to the phenotype of the male father [Sus=100%, Res=0% and Sus<Res or Res<Sus=50%].

CAPÍTULO 3

CANNIBALISM AND INTRAGUILD PREDATION IN REGARDS OF INFERTILE EGGS PRODUCTION BY *Eriopis connexa* (GERMAR) (COLEOPTERA: COCCINELLIDAE)[1]

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[1] Guedes, A.C.S. Cannibalism and intraguild predation in regards of infertile eggs production by *Eriopis connexa* (Germar) (Coleoptera: Coccinellidae). To be submitted.

CANIBALISMO E PREDAÇÃO INTRAGUILDA DE OVOS EM *Eriopis connexa* (GERMAR) (COLEOPTERA: COCCINELLIDAE) RELATIVO À PRODUÇÃO DE OVOS INFÉRTEIS

RESUMO - A oogênese e o desenvolvimento dos ovos são processos de alta demanda de energia para as fêmeas, o que sugere que todos os ovos devem gerar descendentes. Contudo, fêmeas de *Eriopis connexa* (Germar) depositam ovos inférteis, mesmo quando continuamente pareadas com machos viáveis e alimentação abundante. Assim, testamos as hipóteses de que a deposição de ovos inférteis pode variar em função do fenótipo para resistência a inseticidas, frente ao custo reprodutivo imposto à espécie por esta característica (i), o risco de escassez de alimento influencia a deposição de ovos inférteis (ii), e o canibalismo e a predação intraguilda (IGP) de ovos promovem melhorias no desempenho da espécie (iii). A deposição de ovos inférteis foi, em média, de 21.8 e 34.8%, independentemente do fenótipo da fêmea e do tamanho da postura. Períodos de escassez de presa alteraram significativamente a fecundidade, mas não atuou como pista para maior deposição de ovos inférteis. Fêmeas criadas sob canibalismo não tiveram o desempenho melhorado. Contudo, o canibalismo e a IGP como complemento de dieta propiciaram melhorias na fecundidade. O canibalismo e a IGP de ovos por larvas neonatas de *E. connexa* foram densidade dependente, com redução de ambos, em alta densidade da presa extraguilda, prevalecendo o comportamento afidófago de *E. connexa.* Portanto, rejeita-se as hipóteses do estudo propostas para a deposição de ovos inférteis, enquanto que o canibalismo e a IGP em ovos têm papel relevante para *E. connexa*, como complemento de dieta.

PALAVRAS CHAVE: História de vida, fertilidade, comportamento maternal, ovos tróficos, escassez de alimento.

ABSTRACT – Oogenesis and egg development are metabolic activities with a high-energy demand for females, suggesting that all eggs developed should be used to produce offspring. Females of *Eriopis connexa* (Germar), however, lay infertile eggs even when continuously paired with fertile males and abundant food. We studied the production of infertile eggs and the role of egg consumption by other females in the species, based on the hypotheses that production of infertile eggs varies as a function of the lady beetle's phenotype for insecticide resistance. Further, given the reproductive costs imposed on the species by this trait, we hypothesize that females use prey shortage as a clue to produce more infertile eggs in order to provide food for newly hatched larvae. Under these conditions, this egg cannibalism and egg intraguild predation (IGP) should improve the species' performance. Contrary to our hypothesis, we found that the beetles' egg infertility rates were similar between susceptible- and insecticide-resistance phenotypes, but that the number of infertile eggs per egg batch correlated with the number of eggs laid, with percentages of infertile eggs from 21.8 and 34.8%, irrespective of egg batch size. Periods of prey deprivation significantly reduced female fecundity but did not stimulate females to produce infertile eggs as sustenance for newly hatched larvae. Further, egg cannibalism and IGP did not enhance female performance. However, life-history traits were improved when larvae and adults engaged in egg cannibalism or egg IGP if combined with the standard prey diet. In conclusion, we rejected our proposed hypotheses about the role of infertile eggs production by *E. connexa*, even though we found that egg cannibalism and IGP played a relevant role as a dietary complement for *E. connexa*.

KEY WORDS: Life history, fertility, maternal behavior, trophic eggs, food shortage.

Introduction

Egg production is an energetically costly process and it would seem adaptive to have a full egg clutch converted efficiently into offspring (Wheeler 1996, Chapman 1998). Offspring production in lady beetles is a trait that is routinely considered when evaluating the impacts of biotic and abiotic factors on female reproductive success, this outcome is correlated to high egg fertility (Omkar & Pathak 2006, Ferreira *et al.* 2013, Lira *et al.* 2016). However, evolutionary outcomes can diverge from this expected result in some cases, for example when females seek to use infertile eggs to nourish themselves (Santi & Maini 2007) or their offspring (Perry & Roitberg 2005). The fact that sometimes lady beetles lay infertile eggs mixed with fertile ones in the clutch has been interpreted as females efforts' to feed their newly hatched larvae (Polis 1981, Perry & Roitberg 2005, 2006), which commonly stay next to the egg remains and consume infertile and unhatched eggs (Banks 1956, Osawa 1989). In *Eriopis connexa* (Germar), females lay infertile eggs even after one or multiple mates with a mature young male (Colares *et al.* 2015, Table 1S).

In environments with food deprivation or intermittent availability, gravid females may sacrifice some potential progeny to benefit others by laying unfertilized eggs. Under such conditions, egg predation (cannibalism) becomes convenient for both parents and offspring (Polis 1981, Perry & Roitberg 2006). This behavior provides newly emerged larvae some nourishment before they find their first prey (Brown 1972). The egg itself is a highly nutritious food. About 90% or more of the egg content is the yolk, which is composed mostly of lipids and proteins in equal proportions (Chapman 1998, Slogett & Lorenz 2008). Studies have shown that consumption of a single egg allows the survival in newly hatched coccinellid larvae for at least four days in *Chilomenes sexmaculatus* (Fabr.), *Eocaria muiri* (Timberlake), and *Harmonia quadripunctata* (Pontoppidan), and for until ≈ 6 days in *Coccinula sinensis* (Weise) (Elnagdy *et al.* 2011). Furthermore, the survival of larvae (F1) produced by adults fed a cannibalistic diet was increased, even though the larvae themselves did not engage in cannibalism (Bayoumy *et al.* 2016). Thus, cannibalism can result in energy and nutritional benefits for cannibals, such as higher rates of development, growth, and survival, in addition to increased body size and better reproductive outputs (Polis 1981). This benefit occurs due to the higher probability of survival in individuals engaging in cannibalism (Eickwort 1973).

From a biological control standpoint, consumption of conspecifics (cannibalism) or heterospecifics [intraguild predation (IGP)], would reduce the biological control value of the species (Honek & Evans 2012, Lucas 2012). However, if cannibalism and IGP enhance individual survival under food shortage or community maintenance under multiple intra- and interspecific interactions, they could be considered as positive factors for biological control. As mentioned before, if the risk of death by starving is imminent for both the cannibal and its victim, then cannibalism is potentially a net positive outcome (Eickwort 1973). In such cases, cannibalism could benefit biological control by helping to maintain the energy flow in the food web and supporting the predator population in periods of prey scarcity (van den Bosch & Gabriel 1997, Ohlberger *et al.* 2012). Nutrients obtained through cannibalism can help sustain older, healthier individuals in a natural enemy population (Polis 1981, Perry & Roitberg 2006). Similarly, intraguild predation allows the most abundant predator species to serve as intraguild prey when the preferential extraguild prey is rare or absent in the area (Lucas & Rosenheim 2011). It would increase the persistence of predator populations in environments characterized by large fluctuations in food availability, as commonly faced by aphidophagous predators, considering the unpredictable fluctuations of aphid densities, their preferred prey.

In *E. connexa*, resistance to pyrethroids entails costs, possibly due to the enhanced enzymatic activity that increases the insecticide detoxification ability of the resistant phenotype. Also, it seems to be associated with energy-use trade-offs between resistance and reproduction (mainly in support of female fecundity) (Ferreira *et al.* 2013). Previous studies have evaluated the reproductive performance of *E. connexa* under different environmental conditions, mainly in terms of fecundity and survival (Ferreira *et al.* 2013, Colares *et al.* 2015, Lira *et al.* 2016, D'Ávila *et al.* 2018, Nascimento *et al.* 2021). However, there is a lack of data concerning the production of infertile eggs by *E. connexa,* since the works use the hatching rate to measure fertility, not counting the unfertilized eggs. Therefore, we hypothesized (1) that resistant and susceptible females of *E. connexa* may differ in their rate of production of infertile eggs, and the ratio of infertile eggs is correlated to clutch size. Furthermore, considering the extra nutritional gain acquired by the consumption of eggs, we hypothesized (2) that the consumption of conspecific (cannibalism) or heterospecific eggs (IGP) by *E. connexa* adults would enhance the performance of those individuals. Finally, we proposed (3) that under conditions of food inconstancy, females experiencing food shortages should use this experience as a clue to lay more infertile eggs to nourish their offspring, in order to support cannibalism to support their offspring's performance. Therefore, in this work we evaluated the production of infertile eggs by females from two populations (pyrethroid susceptible and resistant) of *E. connexa* and the impact of egg cannibalism and intraguild predation (IGP) on predator development and reproductive performance.

Material and Methods

Insects. We used *E. connexa* and *Coleomegilla maculata* DeGeer for the intraguild predation (IGP) bioassays. These species can be found in many crop ecosystems and share the same habitat (Lixa *et al.* 2010, Harterreiten-Souza *et al.* 2012, Rodrigues *et al.* 2013a). Both species lay their eggs in clutches on plant foliage, making them at risk for conspecific and heterospecific larval and adult predation. Colonies of the two lady beetle species were maintained in the "Laboratório de

Controle Biológico" of Universidade Federal Rural de Pernambuco (UFRPE), Brazil, to provide insects for experiments. For *E. connexa*, two populations were used in bioassays: pyrethroid-resistant (EcRes) and a susceptible (EcSus) populations. The EcRes population has been maintained under insecticide pressure for about of 100 generations in the laboratory, and receives periodically resistant individuals from several locations in Brazil that had been identified through testing for insecticide susceptibility (Costa *et al.* 2018). The EcSus population was established from individuals collected from cotton fields in Frei Miguelinho County, Pernambuco State, and it has been supplemented annually with susceptible individuals collected from cotton fields in the same area, after testing for susceptibility. Meanwhile, the colony of *C. maculata* was started with adults collected in cotton and coriander fields located at Frei Miguelinho and Chã Grande Counties, respectively, both in Pernambuco (PE), Brazil.

Colonies of *E. connexa* were reared at 25 ± 2°C and photoperiod of 12:12-h (L:D), whereas *C. maculata* was reared at 22 ± 1°C and photoperiod of 16:8h (L:D). Larvae from both species were reared at a rate of 2-3 larvae per rearing container (80 mL in volume), and fed *ad libitum* with eggs of *Ephestia kuehniella* (Zeller). In the adult stages, both species were reared in groups of ~100 individuals in Plexiglass$^{®}$ cages (30 cm height × 45 cm width × 50 cm length) as per Rodrigues *et al.* (2013b). Crumpled paper towels were placed inside adult rearing cages to provide oviposition sites. As food for adults, an unlimited amount of *E. kuehniella* eggs, a paste of 50:50% honey: yeast, and aphids were provided. The artificial diet was smeared over pieces of scotch tape fixed to the inner cage wall were insects had access. Also, for *C. maculata* adults, water was provided in a moist sponge inside the cage. Larvae were reared individually in container (80 mL in volume), to avoid cannibalism and were fed *E. kuehniella* eggs. For larvae water was provided as droplets on the container lids.

In bioassays were a preferred extraguild prey (aphids) was present, we used the turnip aphid, *Lipaphis pseudobrassicae* (Davis), which we obtained from collard plants cultivated in the Horticulture area of the "Departamento de Agronomia" of UFRPE, when needed.

Frequency of Production of Infertile Eggs by *Eriopis connexa*. This experiment (#1.1) was carried out to measure the frequency of infertile eggs in randomly collected egg batches produced by different females of varying ages from the laboratory rearing colony of *E. connexa*. Egg batch with more than 5 eggs were collected from the rearing colonies, which totaled 50 egg batches from the pyrethroid-resistant (EcRes) population and 47 egg batches from the susceptible (EcSus) population. Batches were randomly collected without the knowledge of the females that laid them, every other day for 30 days covering the period of major oviposition activity for *E. connexa* female lifetime (Ferreira *et al.* 2013). These eggs were incubated in small plastic plate dishes at 25 ± 1^{o}C and 60-70% R.H. Three days after laid, the embryonic development of each egg was evaluated to detect its fertility. Eggs were diagnosed with the aid of a stereomicroscopic 4-10X (Opton^{TM}, Anatomic do Brazil S.A., Cotia, SP).

In the experiment #1.2, we expected that the earlier egg batches produced will represent the maximum investment of females in their reproductive output following a successful mating. In this assay, 86 and 85 egg batches were monitored for embryonic development. These batches were the 2nd and 3rd egg batches respectively, by 86 EcRes females collected at adult emergence and tracked for oviposition events. For this test, we only used egg batches from EcRes females, as there was no difference in egg fertility between the susceptible and resistance populations (colonies), and EcRes is of more interest for biological control purposes. In these 2nd and 3rd egg batches, there were 3 and 1 batches with five or fewer eggs, respectively, and they were discarded from the analysis. The numbers of fertile and infertile eggs within each egg batch were determined with the same procedures as previously described. Since the analysis comparing rates of infertility from 2nd and the 3rd egg batches did not result in any statistical differences, 2nd and 3rd egg batches were pooled to further investigate the pattern of production of infertile eggs by *E. connexa*.

Development of *Eriopis connexa* Larvae that Engaged in Egg Cannibalism or Intraguild Predation (IGP) and Resulting Level of Egg Production in the Subsequent Adults. In this Experiment #2, we investigated the influences of egg cannibalism or IGP on the larval developmental and adult reproductive output of *E. connexa.* We used 3-day-old larvae in this and subsequent experiments because newly emerged larvae had higher mortality rates if isolated immediately. Thus, larvae (aged from 3 days-old to time of pupation) were reared under each of the following five diets: (1) *E. kuehniella* eggs (*Ek* eggs) only, as control; (2) *E. connexa* eggs (*Econ* eggs) only, representing the pure cannibalism treatment; (3) both *Ek* eggs and *Econ* eggs; (4) *C. maculata* eggs (*Cmac* eggs) only, as the IGP treatment; and (5) *Ek* eggs and *Cmac* eggs. For all treatments, *Econ* eggs (cannibalism) or *Cmac* eggs (IGP), respectively, were offered *ad libitum* and the number of each consumed was recorded by counting the total number of uneaten eggs relative to the numbers offered, during a 24h period when the standard food (*E. kuehniella* eggs) was withheld.

These larval diets were offered to larvae for three feeding events set as 3, 7, and 12 days old. To avoid cannibalism from larvae on other larvae or pupae, larvae were reared individually to the adult stage. Data collected were the time required for total development until pupation, the rate of adult emergence (%), the adult fresh body weight at emergence (< 12h old). We evaluated 30, 31, 30, 39, and 38 larvae in the treatments 1 through 5, respectively.

Newly emerged adults were fed the same prey regime used during the larval stage with the first cannibalism and IGP feeding options in the treatments 2 and 3 being presented on day 4 of adulthood. Male and female pairing occurred at day 5 after emergence. Pairings occurred during the photophase period from 8 to 18h. After verifying the occurrence of mating, males were discarded and the females were individualized and monitored for egg production for 20 days. Cannibalism and IGP feeding options were repeated for groups 2 and 3 on days 12 and 24 of adulthood. Oviposition was recorded for 13, 13, 13, 15, and 15 females for treatments 1 to 5, respectively. The number of egg batches produced were counted, and the number of fertile and infertile eggs per egg batch recorded.

Egg Cannibalism and Intraguild Predation by *Eriopis connexa* Larvae. In this experiment #3, we investigated the predatory behavior of *E. connexa* larvae concerning cannibalism and intraguild predation (IGP). To better understand about the benefits of predation upon conspecific or heterospecific eggs, a series of three bioassays measured the effects of cannibalism or IGP by *E. connexa* larvae on their development. For choice tests, 3-day-old larvae were allowed to choose among conspecific eggs (cannibalism), heterospecific eggs (IG prey, *Cmac* eggs), or the turnip aphid, the field prey (Nascimento *et al.* 2021). We tested *E. connexa* larvae because between larvae and adults, larvae are more prone to suffer prey shortage; therefore, cannibalism and IGP are likely to be more common in larval stage, and more often may contribute to larval survival than to adult survival, since adults can move to new areas by flight to escape from prey shortages.

Egg batches produced by both lady beetle species were harvested daily to provide eggs for this study. Papers bearing egg batches were cut into pieces, sealed inside plastic pots, and stored at -5 ^{o}C in a refrigerator until used. Preliminary tests showed that larval preference and consumption of eggs did not vary between frozen and fresh eggs.

Three tests (Experiments #3.1, #3.2, and #3.3), including no-choice and choice tests, were run to assess larval cannibalism and IGP by *E. connexa* larvae with and without the extraguild prey, the turnip aphid. In each test, the same number of prey items of each type was used (e.g., 20 eggs of each lady beetle species, and the turnip aphid). An equal number of lady beetle eggs for each species was obtained by discarding the any excess eggs in a batch or by combining several egg batches. To differentiate the species of each egg batches in the choice tests, the papers bearing the eggs from one lady beetle species were identified by a red dot. Before tests, larvae were starved for 24h to equalize the hunger level.

Prey and predator interactions were observed in Petri dishes (1.5 cm height × 9 cm diameter). Each dish used was lined with a collard leaf disc of the same diameter cut from a fresh collard leaf. On the leaf disc, the egg batches or the aphids were offered as prey.

For Exp. #3.1 (the no-choice test), a single prey type was offered in each dish (i.e., only eggs of *E. connexa*, eggs of *C. maculata*, or aphids) (n = 20 prey per larvae). The aphids were obtained from infested collard leaves harvested from the field and left to wither for 4-5 h in a white plastic tray. Under this condition, most of the aphids abandoned the leaf and started to walk away in the tray, avoided damaging the aphids' mouth parts, and facilitated their collection and transference to the leaf disc inside the dishes. Third instar nymphs of the turnip aphids were transferred to the leaf discs 1h before releasing the predator larvae to allow time to settle.

In Exp. #3.2 (the choice test), three comparisons were offered together to individual *E. connexa* larvae: (1) *Econ* eggs (cannibalism) vs. *Cmac* eggs (IGP) (n = 16 larvae); *Econ* eggs (cannibalism) vs. turnip aphids (extraguild prey) (n = 19 larvae); and (3) *Cmac* eggs (IGP) vs turnip aphids (extraguild prey) (n = 20 larvae). The arena and prey availability were set up similarly to the non-choice test with 20 prey items of each type.

In Exp. #3.3, we used a three-way choice test, in which all three preys were offered simultaneously. There were two subsets of this experiment, in which the aphid density was changed, being either 20 aphids (high) or 5 aphids (low). The control for Exp. #3.3, was to offer the low aphid densities alone (no other prey) (n=16 larvae) to measure the aphid predation rate without any influence of other foods. For each part of Exp. #3.3, we recorded the number of each prey consumed by the larva during 24h from the release of the lady beetle larva.

For a better interpretation of the consumption rates of larvae of *E. connexa*, we measured the size of the eggs offered and used the measurements to calculate the egg volume for each of 20 freshly laid eggs of each species. The egg volume (V) of both *E. connexa* and *C. maculata* were calculated using the formula $V = \pi LW^2/6$, where L and W stand for length and width (Takakura 2004), adapted from the ellipsoid volume formula [$V = 4/3\pi a(b^2)$] where "a" and "b" stand for radius of the length and the width. To obtain the needed measurements, eggs, on their day of oviposition, were aligned on a Petri dish, lined with filter paper, and photographed with a digital camera attached to a stereomicroscope. Images were imported into the software AutoCAD® (Autodesk® Inc. 2020) and adjusted to 1: 1 scale, using the SCALE command. The measures of L and W were obtained by applying the command COTA, according to the methodology adapted from Damasceno *et al.* (2015).

The Risk of Food Shortage and Production of Infertile Eggs in *Eriopis connexa*. If infertile eggs are produced to improve food availability for newly emerged larvae, experiencing a food shortage should lead to increased production of infertile eggs. Thus, in this experiment (#4), we tested the hypothesis that prey shortage and cannibalism play a role in a female decision on laying infertile eggs to guarantee nourishment the new larvae. We measured the impact of food deprivation by larvae or adults on the level of production of infertile eggs by females when adults were also allowed to cannibalize their own eggs, compared to larvae and adults fed *ad libitum*. Thus, we allowed larvae to experience food shortage and cannibalism. Then, the adults were kept on the same diet and starvation regime as their larval group to see if these environmental conditions increased infertile egg production by adult females. Larvae (3 days-old) were reared until pupation and then as adults under a dietary regime of inconstancy of food availability. The prey deprivation condition was set as 48h without any type of food followed by 24h of *ad libitum* feeding.

For this experiment, we run a total of four treatments including the control treatment with larvae and adults fed continuously on *Ek* eggs (1). In treatments (2-4 below), larvae and adults were fed regimes that incorporated some degree of prey deprivation, followed by opportunities for cannibalism or intraguild predation. This form of prey deprivation was applied to three treatments in which the diet varied: (2) larvae and adults fed only the standard rearing prey *Ek* eggs, (3) larvae and adults fed only *Econ* eggs (i.e., cannibalism), and (4) larvae and adults that were alternately fed *Econ* eggs and then *Ek* eggs (i.e., predation and cannibalism). All larvae were reared individually across all treatments to avoid cannibalism from larvae upon larvae or upon pupae.

Data were collected for *E. connexa* for each of the treatments and the control for the time for pupation, rate of adult emergence (%), fresh body weight at adult emergence (<12h old) for mixed sexes (adult *E. connexa* do not exhibit any sex morphological dimorphism). For each treatment, we started the experiment with 46 larvae, and each larva was considered as one replicate.

Newly emerged adults were reared on the same food regime (i.e., 48h without any type of food followed by 24h of *ad libitum* feeding) similar to their larval stage. Adults were paired at 5 days-old. After verification of mating, males were discarded to avoid male cannibalism on eggs. Females were monitored daily for egg production and egg batched removed daily. For each egg batch, we recorded the number of fertile and infertile eggs determined as previously described for Exp. #1. Ultimately, data were obtained on 17, 15, 11, and 17 females for the treatment control (*Ek* eggs *ad libitum*), prey shortage when fed *Ek* eggs, prey shortage when fed *Econ* eggs (i.e., cannibalism treatment), and prey shortage when alternately fed *Econ* eggs (cannibalism) or *Ek* eggs.

Data Analysis. The number of eggs per egg batch, the number of infertile eggs, and the percentage of infertile eggs in the egg batch were compared between pyrethroid-resistant (EcRes) and susceptible (EcSus) populations for both egg batch experiments (#1.1 and #1.2) by student t-test using the PROC TTEST of SAS (SAS Institute 2002). This analysis indicated a lack of population effect ($P > 0.05$) on these characteristics, subsequently the following analysis disregarded the population effect. Thus, the relationship of the number of infertile eggs produced as a function of the total number of eggs per batch was calculated using the regression analysis performed by PROC REG of SAS (SAS Institute 2002). The model selected yielded an equation with significant parameters ($P < 0.05$) and with the greatest adjusted coefficient of correlation (r) for the egg batch experiment.

In the Experiment #2, the duration period of larval and pupal stages, the period from larval emergence to adult and fresh body weight at adult eclosion did not fit the ANOVA assumptions despite data transformation. Thus, these data were analyzed with Kruskal-Wallis's test for multiple comparisons ($\alpha = 0.05$), followed by Mann-Whitney-Wilcoxon Rank Sum test for more than two means, using 'R' and the package 'agricolae' (R Core Team 2013). Fecundity data were subjected to tests for normality (Shapiro-Wilk) and homogeneity of variance (Bartlett), and subjected to one-way ANOVA and Tukey HSD's test ($\alpha = 0.05$) for mean separation. The percent of infertile eggs did not fit the ANOVA assumptions despite data transformation, and were analyzed with Kruskal-Wallis test to the multiple comparison ($\alpha = 0.05$), followed by Mann-Whitney-Wilcoxon Rank Sum test for more than two groups, using 'R' and the package 'agricolae' (R Core Team 2013). The survival of the larval and adult stages was calculated using the Kaplan–Meier method, and the survival means were compared through the LogRank's test using PROC LIFETEST of SAS (SAS Institute 2002).

Data on predation in the non-choice test, run with three prey types separately (experiment #3.1) regarding egg cannibalism and IGP, or when caged with high and low aphid density (in the experiment #3.3) were square root (x + 0.5) transformed to fit the ANOVA assumptions, and means comparisons by one-way ANOVA followed by Tukey's Honestly Significant Difference test (α = 0.05), or submitted to PROC TTEST for two preys under the pooled method for equality of variance. Otherwise, the data from choice tests were compared under the statistical hypothesis of equal 50%:50% output between two prey items (experiment #3.2) or 33%:33%:33% output across three prey items (experiment #3.3). The comparisons were performed using the PROC FREQ of SAS (SAS Institute 2002) and interpreted through chi-square test (α = 0.05).

In the experiment #4, none of the parameters evaluated fit the ANOVA assumptions despite data transformation. Thus, these data were analyzed with Kruskal-Wallis's test for multiple comparisons (α = 0.05), followed by Mann-Whitney-Wilcoxon Rank Sum test for more than two means, using 'R' and the package 'agricolae' (R Core Team 2013).

The egg volume data were transformed into square root (x + 0.5) to satisfy the assumptions of normality and homoscedasticity, which were verified using the PROC UNIVARIATE from SAS. In addition, differences on egg volume were tested with Student t-tests using PROC TTEST with the Satterthwaite method for equality of variance (SAS Institute 2002).

Results

Frequency of Production of Infertile Eggs by *Eriopis connexa*. There was no significant difference in the frequency of production of infertile eggs between pesticide resistant (EcRes) and insecticide susceptible (EcSus) *E. connexa* females (Exp. #1.1). Similarly, there was no significant difference between the rates of infertile eggs in a female's 2nd vs. 3rd egg batches per lifetime (Exp. #.2) (Table 1). Furthermore, the mean number of eggs and infertile eggs per batch and the proportion of the infertile eggs per batch were also similar between the studied populations and egg batches (Table 1). Thus, these data were pooled to infer the production of infertile eggs by *E. connexa* females.

The number of infertile eggs per batch increased significantly as a function of the number of eggs per batch in both Exp. #1.1 and #1.2 (Figs. 1A and B) because females maintained a similar percentage of infertile eggs laid per egg batch (Fig. 1A' and B'). On average there were 34.8 and 21.8% infertile eggs for batches randomly collected from the rearing colony and for batches produced as the 2nd or 3rd ones in a female's life, irrespective of the batch size.

Development of *Eriopis connexa* Larvae that Engaged in Egg Cannibalism or Intraguild Predation (IGP) and Resulting Level of Egg Production in the Subsequent Adults. Prey treatment significantly affected *E. connexa* larval developmental time ($H = 137.67$, $df = 4$, $P < 0.0001$) and adult fresh body weight at emergence ($H = 101.78$, $df = 3$, $P < 0.0001$) (Table 2). The duration of larval development varied from 10.7 to 14.1 days, with larvae fed *Cmac* eggs alone or combined with *Ek* eggs developing faster; while *Econ* eggs resulted in ~4 days of development delay, followed by the combination of *Ek* and *Econ* eggs with ~3 days delay in development. Nonetheless, faster development of larvae fed *Cmac* eggs alone resulted in smaller adults compared to adult's originated from larvae fed *Ek* and *Econ* eggs or larvae fed *Ek* eggs alone (Table 2).

Females fed *Cmac* eggs, *Cmac* + *Ek* eggs or *Econ* + *Ek* eggs had higher fecundity than females fed *Econ* eggs only or *Ek* eggs only (Table 2). On the other hand, egg fertility and survival rates were unaltered by these treatments. Females fed *Ek* eggs had lower fecundity for the

20-day evaluation period compared to females whose diet was supplemented by *Econ* eggs or *Cmac* eggs (Table 2).

Larvae and adults of *E. connexa* eating conspecific (*Econ*) and heterospecific (*Cmac*) eggs in both the presence and absence of standard prey (*Ek* eggs), but they varied the number of eggs consumed as a function of egg availability and the age of *E. connexa* (Table 3). *Eriopis connexa* larvae that were 3 days old consumed similar numbers of *Econ* and *Cmac* eggs when they were offered separately, but consumed fewer *Cmac* eggs (IGP) when they were offered together with the *Ek* eggs. However, 7-day-old larvae and adults at 12 days after mating consumed more *Econ* eggs than *Cmac* eggs when they were offered separately (Table 3). Likewise, larvae also consumed more *Cmac* eggs when they were offered together with the *Ek* eggs. The adult female, however, switched the egg consumption by consuming more *Cmac* than *Econ* eggs when the *Ek* eggs was also present.

Egg cannibalism and Intraguild Predation by *Eriopis connexa* larvae. The numbers of conspecific (*Econ*) or heterospecific (*Cmac*) eggs consumed by 3-day-old larvae, during a 24h period under no choice conditions (Exp. #3.1), were greater than the number of the turnip aphids (the preferred prey) ($F_{2, 74} = 11.79$, $P < 0.0001$) (Fig. 2A). The average daily consumption was 8.6, 6.6, and 3.8 *Econ* eggs (cannibalism), *Cmac* eggs (intraguild), or aphids (extraguild), respectively.

Under choice test conditions (Exp. #3.2), there was no difference between consumption of *Econ* eggs vs. *Cmac* eggs ($\chi^2 = 1.79$, $P = 0.18$), and *Econ* eggs vs. aphids ($\chi^2 = 0.84$, $P = 0.36$) (Fig. 2B). However, 3-day-old larvae preferred to consume significantly more the aphids vs. *Cmac* eggs ($\chi^2 = 15.78$, $P < 0.0001$). Specifically, larvae consumed 2.1 *Cmac* eggs and 5.5 aphids, in 24h test period.

The density of aphids (Exp. #3.3), affected the number of the other prey items (*Econ* eggs or *Cmac* eggs) consumed (aphids at 5 vs. 20 individuals) (t-test = 6.71, $P < 0.0001$, df = 29) (Fig.

3). Increasing the number of aphids from 5 to 20 aphids resulted in almost 2-fold greater aphid consumption at the higher aphid density (2.3 vs 5.7 aphids), but the percentage of aphids consumed declined, indicating that the higher extraguild prey density (ca. 20 aphids) resulted in a surplus of prey for larvae. Lower numbers of turnip aphids [which is one preferred extraguild prey (Lira *et al*. 2019)] in this choice test, resulted in a significant increase in cannibalism upon *Econ* eggs ($\chi^2 = 13.31$, $P = 0.0013$) (Fig. 3). On the other hand, aphids were more consumed in higher availability ($\chi^2 = 25.41$, $P < 0.0001$), with reduction of cannibalism. At the low density of aphids, there was a consumption of 4.8 *Econ* eggs, compared to 2.3 *Cmac* eggs, and 1.7 aphids. However, when aphids were offered at higher density (20 aphids), more aphids were consumed (average of 4.9) and that diet was supplemented with some others, but fewer, preys (1.5 and 1.1 *Econ* and *Cmac* eggs, respectively).

Since prey varied in size, we also considered prey quantity as total volume consumed. The estimated volumes of the studied lady beetle species indicated that *Cmac* eggs are larger (df = 30.6, t = 10.56, $P < 0.0001$) than that of *Econ* eggs. The average volume of *Cmac* eggs was 0.243 (± 0.007) mm^3 while for *Econ* eggs was 0.152 (± 0.004) mm^3 (Fig. 4). Based on that, a single *Cmac* egg represents 37.4% more prey content than that on *Econ* eggs. Nonetheless, the *Econ* larvae did not exhibit any difference between cannibalism and intraguild prey consumption in choice ($\chi^2 = 1.79$, $P = 0.1808$) and non-choice tests (df = 30, t = 1.33, $P = 0.1937$).

The Risk of Food Shortage and Production of Infertile Eggs in *Eriopis connexa*. Larva and adult traits of *E. connexa* were altered when subjected to 48h prey deprivation followed by 24h prey availability (Table 4). Larvae fed continuously on *Ek* eggs, exhibited shorter developmental time, greater adult fresh body weight at emergence day compared to treatments with 48h prey deprivation in the larval stage. Furthermore, the option of cannibalism under prey deprivation (feeding *Econ* eggs) instead of improving performance caused a delay of 6 days in larval development and 5.8 days for complete development from larvae to adult emergence. In addition, larvae under an exclusive cannibalistic diet exhibited reduced survival (71.7%), compared to 91.3 or 100% under the other diets.

Adult females reared under prey deprivation had similar survival within the 20 days adulthood that were observed, with 100% survival across all treatments. Nevertheless, prey deprivation caused a drastic reduction in egg production for all prey combinations (Table 4). Two females from the control treatment (females fed continuously with *Ek* eggs) were infertile; their data were excluded from the analysis of fecundity and egg fertility. Females reared exclusive under cannibalism exhibited the largest reduction in egg production. Females without prey deprivation (control) produced 3.5x, 4.7x, and 13.6x more eggs than females reared under 48h prey deprivation fed (1) *Ek* eggs, (2) alternating between cannibalism and *Ek* eggs, and (3) *E. connexa* eggs, respectively (Table 4). Besides producing fewer eggs compared to females in the other treatments, females fed the cannibalistic diet also deposited more infertile eggs per egg batch, compared to females in the other treatments (Table 4).

Discussion

The production of infertile eggs by *E. connexa* occurred at similar rates in both susceptible and pyrethroids resistant populations, regardless of when the egg batch was laid within the female's lifetime. However, in all cases, the total number of infertile eggs increased with increasing the numbers of eggs per batch. This pattern of there being more infertile eggs in larger egg batches suggests that with larger egg batches, there would be higher costs to support food (egg) provisioning for newly emerged larvae. Irrespective of the size of the egg batches, ~21 to 34% of the eggs laid were infertile (Fig. 1). This high proportion of infertile eggs for *E. connexa* has been frequently noted previously. From other studies, it can been seen that infertility varies from 15.5% (Zazyckia *et al*. 2015) to 75.4% (Ferreira *et al.* 2013) (Table 1S). Production of infertile eggs by *E. connexa* is not related to lack of sperm (Colares *et al*. 2015, Silva *et al.* 2021). However, the reason for this oviposition pattern remains unclear. Nevertheless, it is a common feature of the species, since only a small portion (5.8%) of egg batches in *E. connexa* (Exps. #1.1 and #1.2) exhibited 100% egg fertility.

In phytophagous species held without food restriction, production of infertile eggs is usually low, with fertility being near 100% in most of the cases (Saeed *et al.* 2010, Pacheco da Silva *et al.* 2017), consistent with the fact that egg production is costly and eggs should be converted efficiently into offspring. In the case studied here, of a resistant and a susceptible lady beetle population, low fertility is a negative influence given that the maintenance of the resistant phenotype requires that sufficient individuals of the resistant phenotype be produced (Rodrigues *et al.* 2013b), so that the resistant population will be maintained over successive generations (Rodrigues *et al.* 2020). This maintenance depends fundamentally on the reproductive performance of the resistant population’s offspring (Lira *et al.* 2016). High levels of infertile eggs production works against the population growth, but it may enhance the probability of survival of newly emerged larvae justifying the level of consistence in infertile eggs production.

Prey type can affect the efficiency (Ingested Material Conversion Efficiency, IMCE) with which food is converted to eggs in lady beetles (Honek & Evans 2012). In our system, larvae of *E. connexa* developed faster when fed *C. maculata* eggs (an intraguild prey), but did not when fed their own eggs (cannibalism). This reduction in larval developmental time when fed the IGP (*C. maculata* eggs) cannot be generalized and deserve further studies using eggs of other lady beetle species to certify the benefits of IGP over cannibalism, especially, due to difference on chemical compounds (nutrients and semiochemicals) of lady beetle eggs. Lady beetles eggs contain defense compounds against predation (Hemptinne *et al.* 2000, Sloggett & Davis 2010, Katsanis *et al.*

2017), which likely vary by lady beetle species and the metabolism of these compounds may impose some costs. For instance, the lady beetle *Harmonia axyridis* (Pallas) possesses a specialized ability to metabolize alkaloids, which is found in most lady beetle species (Sloggett & Davis 2010). However, *C. maculata* eggs are toxic to *H. axyridis* (Sloggett *et al.* 2009). In *E. connexa* larvae, it is probable that alkaloid metabolism obtained from *C. maculata* eggs diverted energy from adult biomass production, resulting in faster larval development, but resulting in smaller adults. Moreover, it may have occurred because *C. maculata* eggs are larger, which translates into more energy per prey item consumed compared to the conspecific eggs.

Interestingly cannibalism resulted in a delay in the development, contrary to *Hippodamia convergens* Guerin-Meneville, where cannibal individuals developed faster (Bayoumy & Michaud 2015). However, *E. connexa* larvae fed their own species' eggs attained a final adult body fresh weight that was similar to the control group fed *E. kuehniella* eggs, possibly because any egg defense compounds would be similar to their own, and thus pose no important toxicological issues for larval development.

The nutritional requirements of insects vary depending on the energy demand of each life stage (i.e., development and reproduction), and adult females have a greater need of protein for oogenesis (Chapman 1998). Egg consumption (as cannibalism or IGP) increased the fecundity of *E. connexa* adults because eggs are an efficient source of protein for oogenesis. This benefit did not occur when adults of *E. connexa* were fed larvae of diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae), which proved to be nutritionally unsuitable to this species (Nascimento *et al.* 2021). The nutritious value of foods, however, is variable. While the cannibalism of a single egg at a first larval stage may be sufficient to improve biological parameters larvae of *H. axyridis* (Osawa 2002), a diet of *E. kuehniella* eggs complemented with two eggs (cannibalism) did not improve either development or reproduction of *C. maculata*, perhaps due to fewer adaptations (behavioral or physiological) associated with egg cannibalism (Abdelwahab *et al.* 2018). Indeed, fecundity even decreased when larvae of *Menochilus sexmaculatus* Fabr. were reared feed exclusively with eggs of their own species (Yadav *et al.* 2020). Our results support the general view that both egg cannibalism and IGP have benefits to *E. connexa*, but that these benefits are different between larvae and adults.

Eriopis connexa shares habitat and prey species in the crop ecosystems with other lady beetles (Lixa *et al.* 2010, Harterreiten-Souza *et al.* 2012, Rodrigues *et al.* 2013a). This overlap creates a wide range of potential intra- and interspecific interactions, such as cannibalism and IGP. Overall, both cannibalism and IGP occur when larger (older) individuals/species prey on smaller (younger) individuals/species (Polis 1981, Polis *et al.* 1989). In IGP, the degree of trophic specialization is an important factor influencing the frequency and direction of the interaction (Polis *et al.* 1989). For cannibalism, prey abundance is a key factor with more cannibalism occurring when prey is scarce. For example, first instar larvae of *T. notata* and *C. montrouzieri* show high rates of cannibalism, around 65% and 55%, respectively, under prey scarcity (Oliveira 2020). This relationship acts as a buffer to limit the impact of variable food resources, adjusting the lady beetle population size to the existing resources (Polis 1981). IGP is more complex but also increases with reducing availability of extraguild prey, driving the hungry predators to expand their diet to include intraguild prey (Polis *et al.* 1989). In *E. connexa* larvae, we found that in the absence of aphids, both cannibalism and IGP increased, indicating that *E. connexa* larvae would use these foods to survive in the field during periods of scarcity of aphids. Even in the presence of aphids, *E. connexa* larvae engaged in egg cannibalism, showing its value as a source of extra or unique nutrients that were not obtained from eating only aphids. However, *E. connexa* demonstrated a preference for aphids over IGP. This preference for aphids (extraguild prey) may be related to defense compounds present in *C. maculata* eggs (Sloggett *et al.* 2009), how suggests the intense yellow color from these eggs, indicating large amounts of alkaloids, making these eggs less attractive or less palatable, since the more alkaloids in a species, more unpalatable it becomes (Pasteels *et al*. 1973). Secondly, *C. maculata* eggs are larger than either *E. connexa* eggs or turnip aphids; therefore, one *C. maculata* egg would promote satiety faster than consumption of the same number of prey items through cannibalism or aphid consumption. Regardless of prey availability, *E. connexa* consistently engages in egg cannibalism and IGP, which implies that these food items are an important complement to aphid diet. This shows the aphidophagous feeding behavior of *E. connexa* but also emphasizes the importance to this species of a mix prey diet (Lira *et al.* 2019, Nascimento *et al.* 2021) and of non-prey food (Santos *et al.* 2016).

When *E. connexa* larvae were exposed simultaneously to extraguild prey in low density, intraguild prey, and cannibalism, their preference for cannibalism shows that conspecific eggs were a better food source than IGP. However, larvae clearly showed a preference towards eating aphids (the extraguild prey) when they were abundant. Nevertheless, larvae continued to engage in cannibalism and IGP (albeit at lower levels), what confirms the density-dependent nature of the influence of extraguild prey on levels of cannibalism and IGP (Lucas & Brodeur 1999). Furthermore, *E. connexa* exhibits a functional response type II in response to change in prey density (Sarmento *et al.* 2007, Lira *et al.* 2019, Santos *et al.* 2021). Therefore, it is expected that an increase in the abundance of an extraguild prey would result in more predation, but the lady beetle's response toward to the target pest (aphids, the extraguild prey) will depend on levels of other possible prey and food sources in the habitat.

In the field, aphidophagous lady beetles often face wide variation in prey density (Kindlmann *et al.* 2015), and in these situations they may turn to alternative foods (Giorgi *et al.* 2009, Lundgren 2009) or cannibalism (Polis 1981). To avoid starvation, coccinellids such as *E. connexa* often consume other non-prey foods (such as nectar, honeydew, pollen, sap, fruit, plants, and fungi (Castro-Guedes 2013, Santos *et al.* 2016). In *E. connexa*, all individuals that experienced food deprivation had delayed development, but those reared only under cannibalism, a protein-rich diet, took longer to the standard prey to reach the adult stage and showed poor reproductive performance, highlighting the importance of a mixed diet (Castro-Guedes *et al.* 2016, Nascimento *et al*. 2021). Furthermore, individuals subjected to prey deprivation had reduced fecundity, and again, an exclusively cannibalistic diet gave the poorest result, in contradiction to our hypothesis that *E. connexa* females lay infertile eggs to nourish their offspring and cannibalism would enhance larval survival. Furthermore, larvae and adults of *E. connexa* have different nutritional requirements. Although both stages consume alternative food (both prey and non-prey items), not all these foods are nutritionally adequate for both development and reproduction (Giorgi *et al.* 2009, Lundgren 2009, Lima *et al.* 2020). Despite the high lipid and protein content of eggs, they lack other nutrients (Chapman 1998, Slogett & Lorenz 2008), essential for metabolism (Lundgren 2009).

Dissected females had malformation of the reproductive organs and poor development of the ovarioles. The poor reproductive performance of females reared exclusively on a cannibalistic diet indicates that diet did not provide sufficient energy for development of the reproductive organs. A similar result with *E. connexa* was found by Nascimento *et al.* (2021), who observed that females fed exclusively with larvae of diamond back moth, *P. xylostella*, did not produce eggs, suggesting that failed to mature reproductively. However, these authors did not perform morphological studies, and they attributed the failure to produce normal numbers of eggs to a poor nutritional value of *P. xylostella* larvae.

Indeed, cannibalism in general should not be considered a reproductive strategy, but rather a survival strategy in times of prey scarcity (Polis 1981, Perry & Roitberg 2006) or, perhaps, a self-regulation mechanism acting to reduce the populations growth potential (Cushing 1992).

Thus, cannibalistic populations can self-regulate below the carrying capacity of the environment, before high density produces over exploitation and mass starvation (Polis 1981). In this context, cannibalism can be understood as a strategy to both sustain a population in the field and stabilize its density in the environment.

In summary, we found that the production of infertile eggs did not vary between the two *E. connexa* phenotypes studied (resistant and susceptible), but rather females from both phenotypes produced a stable proportion of infertile eggs in egg batches, independent of egg batch size. Among the foods tested to preserve *E. connexa* during periods of starvation intraguild predation reduced total developmental time, but egg cannibalism did not. Both cannibalism and IGP, completing the standard diet, increased the adult fecundity but not change the rate of egg fertility. The density of the extraguild prey (turnip aphid) influenced both intra and interspecific interactions, and at high extraguild prey density, both cannibalism and IGP declined (3.2 and 2.2 times, respectively), highlighting the fundamentally aphidophagous nature of *E. connexa*. Starvation did not stimulate females of *E. connexa* to produce more infertile eggs, and cannibalism alone did not enhance the fecundity. In conclusion, we have not explained why *E. connexa* produces infertile eggs at such high rates, and clarify of the mechanism behind this phenomenon will have to be addressed in future studies.

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Table 1. Egg batch size and egg infertility rates by *Eriopis connexa* (A) from a pyrethroid-resistant (EcRes) or a pesticide-susceptible (EcSus) population from the laboratory rearing colonies or (B) from 2nd- vs. 3rd-egg batches produced in a female's lifespan. Note: mean values with minimum and maximum values within bracts.

Egg batches	No. of egg batches analyzed	Mean eggs per batch (min-max)	Mean number of infertile eggs/batch	% infertile eggs
A. Rearing colony				
EcRes	50	20.7 (8 – 36)	6.8 (1 -19)	34.6 (4.5 – 93.3)
EcSus	47	17.7 (6 – 46)	7.4 (1 – 29)	42.7 (5.8 – 93.5)
Mean		19.2 (6 – 46)	7.1 (1 – 29)	34.8 (4.5 – 93.5)
$\textit{t-test}_{df=95}$		$1.95^{0.056}$	$-0.31^{0.753}$	$-1.73^{0.086}$
B. Selected egg batches				
2nd egg batch	78	21.8 (6 – 50)	5.3 (1 – 18)	22.8 (2.8 – 69.2)
3rd egg batch	79	24.2 (6 – 57)	4.9 (1 - 19)	20.9 (2.9 – 64.7)
Mean		23.4 (6 – 57)	5.1 (1 – 19)	21.8 (2.8 – 69.8)
$\textit{t-test}_{df=155}$		$1.20^{0.239}$	$0.69^{0.496}$	$0.80^{0.423}$

Table 2. Life history characteristics (mean ± SE) of immature stages and adults of *Eriopis connexa* fed the standard rearing prey [*Ephestia kuehniella* eggs continuously (*Ek*)] or *Ek* eggs plus occasional access to conspecific [*Eriopis connexa* eggs (*Econ*)], or heterospecific eggs [(*Coelomegulla maculata* eggs (*Cmac*)].

	Prey availability					Statistics		
Characteristics[1]	*Ek* eggs (n =13)[2]	*Econ* eggs (n =13)	*Ek* + *Econ* eggs (n =13)	*Cmac* eggs (n =15)	*Ek* + *Cmac* (n =15)	*F* or *H*	*df*	*P*
Immature stages								
Duration of larval stage (days)**	12.7 ± 0.16 c	14.1 ± 0.25 a	13.3 ± 0.17 b	10.8 ± 0.09 d	10.8 ± 0.07 d	137.67	4	< 0.0001
Duration of pupal stage (days)**	3.3 ± 0.10	3.5 ± 0.09	3.7 ± 0.10	3.6 ± 0.09	3.5 ± 0.08	8.38	4	0.079
Viability of larvae to adult (%)***	100	100	100	97.6 ± 2.41	97.5 ± 2.47	2.28	4	0.69
Adult fresh body weight (mg)**	11.7 ± 0.69 ab	10.9 ± 0.49 ab	12.1 ± 0.55 a	8.8 ± 0.24 c	9.6 ± 0.23 bc	22.13	4	< 0.0001
Adult stage[2]								
No. of eggs/female*	171.9 ± 26.67 b	276.7 ± 32.72 b	309.1 ± 35.56 a	326.9 ± 32.04 a	327.6 ± 28.94 a	4.26	4	0.0041
Fertile eggs (%)**	67.6 ± 6.29	67.8 ± 3.81	62.1 ± 7.72	62.8 ± 7.33	54.8 ± 6.95	2.43	4	0.6585
Survival for 20 days (%)***	100	100	100	93.7 ± 6.05	100	3.37	4	0.4971

[1] Analysis by one-way ANOVA (*), or Kruskal-Wallis (**) or Log-Rank Test (***). Means within rows followed by different letters were significantly different (ANOVA: Tukey HSD’s test; and Kruskal-Wallis: Wilcoxon pairwise test. α = 0.05). [2] Number of females monitored for 20 days observation period.

Table 3. Number (mean ± SE) of eggs consumed during 24h availability period by larvae and adults of *Eriopis connexa* fed (1) the standard rearing prey [*Ephestia kuehniella* eggs (*Ek*)], (2) *Ek* eggs plus occasional conspecific eggs [*Eriopis connexa* (*Econ*)], or (3) heterospecific eggs [(*Coelomegulla maculata* (*Cmac*)], each at different points (age in days) in their lives.

	Age of larvae[2]		Age of adults	
Prey availability[1]	3 days-old	Day 7	Day 4	Day 12
Econ eggs	11.2 ± 1.10 a	34.7 ± 1.03 a	51.6 ± 0.56 a	69.7 ± 1.49 a
Ek + *Econ*	10.3 ± 0.97 ab	20.5 ± 1.08 b	19.2 ± 0.34 d	34.0 ± 0.95 d
Cmac eggs	11.9 ± 0.35 a	20.9 ± 0.70 b	40.4 ± 1.92 b	60.9 ± 1.77 b
Ek + *Cmac*	7.7 ± 0.44 b	15.2 ± 0.80 c	25.1 ± 2.51 c	42.1 ± 4.02 c
Statistics	29.18 $^{(< 0.0001)}$	96.98 $^{(< 0.0001)}$	45.22 $^{(< 0.0001)}$	41.77 $^{(< 0.0001)}$

[1] Analysis by Kruskal-Wallis. Means within columns followed by different letters were significantly different (Kruskal-Wallis: Wilcoxon pairwise test, α = 0.05).

[2] Most of larvae was in pre-pupa or pupal stage in the Day 12 of the feeding event lacking data for analysis.

Table 4. Life history characteristics (mean ± SE) of immatures and adults of *Eriopis connexa* fed *Ephestia kuehniella* eggs (*Ek*) or subjected to prey deprivation (48h, followed by 24h with abundant prey), in each of three diets: *Ek* eggs (control), conspecific eggs (*Econ*), or alternation of *Ek*/*Econ* eggs.

		48h of prey deprivation			Statistics		
Characteristics[1]	Standard prey (*Ek*) daily (n =17)[2]	Standard prey (*Ek*) (n =15)	Conspecific eggs (*Econ*) (n =11)	*Ek* and *Econ* alternately (n =17)	*F*, *H* or χ^2	*df*	*P*
Immature stages							
Duration of larval stage (days)*	11.4 ± 0.09 c	14.9 ± 0.30 b	17.2 ± 0.41 a	14.5 ± 0.18 b	122.77	3	< 0.0001
Duration of pupal stage (days)*	3.6 ± 0.07 c	3.9 ± 0.08 ab	3.7 ± 0.08 bc	3.9 ± 0.05 a	10.03	3	0.0183
Time to reach adult stage (days)*	15.1 ± 0.07 c	18.8 ± 0.31 b	20.9 ± 0.39 a	18.4 ± 0.15 b	123.82	3	< 0.0001
% survival (larvae to adult)**	100 a	91.3 ± 4.15 b	71.7 ± 6.64 c	100 a	26.27	3	< 0.0001
Adult fresh body weight (mg)*	11.7 ± 0.38 a	5.4 ± 0.16 c	6.1 ± 0.28 b	6.4 ± 0.17 b	101.78	3	< 0.0001
Adult stage[2]							
No. of eggs/female*	463.2 ± 37.92 a	107.6 ± 11.95 b	37.7 ± 7.71 c	94.8 ± 9.47 b	44.17	3	< 0.0001
Fertile eggs (%)*	50.1 ± 6.92 a	64.8 ± 7.42 a	19.6 ± 10.15 b	59.0 ± 7.50 a	12.46	3	0.0059

[1] Analysis by Kruskal-Wallis (*) or Log-Rank Test (**). Means within rows followed by different letters were significantly different (Kruskal-Wallis: Wilcoxon pairwise test, α = 0.05, Kaplan-Meier Survival: Log-Rank).

[2] Number of females monitored for 20 days and all ended the observation period alive (100%).

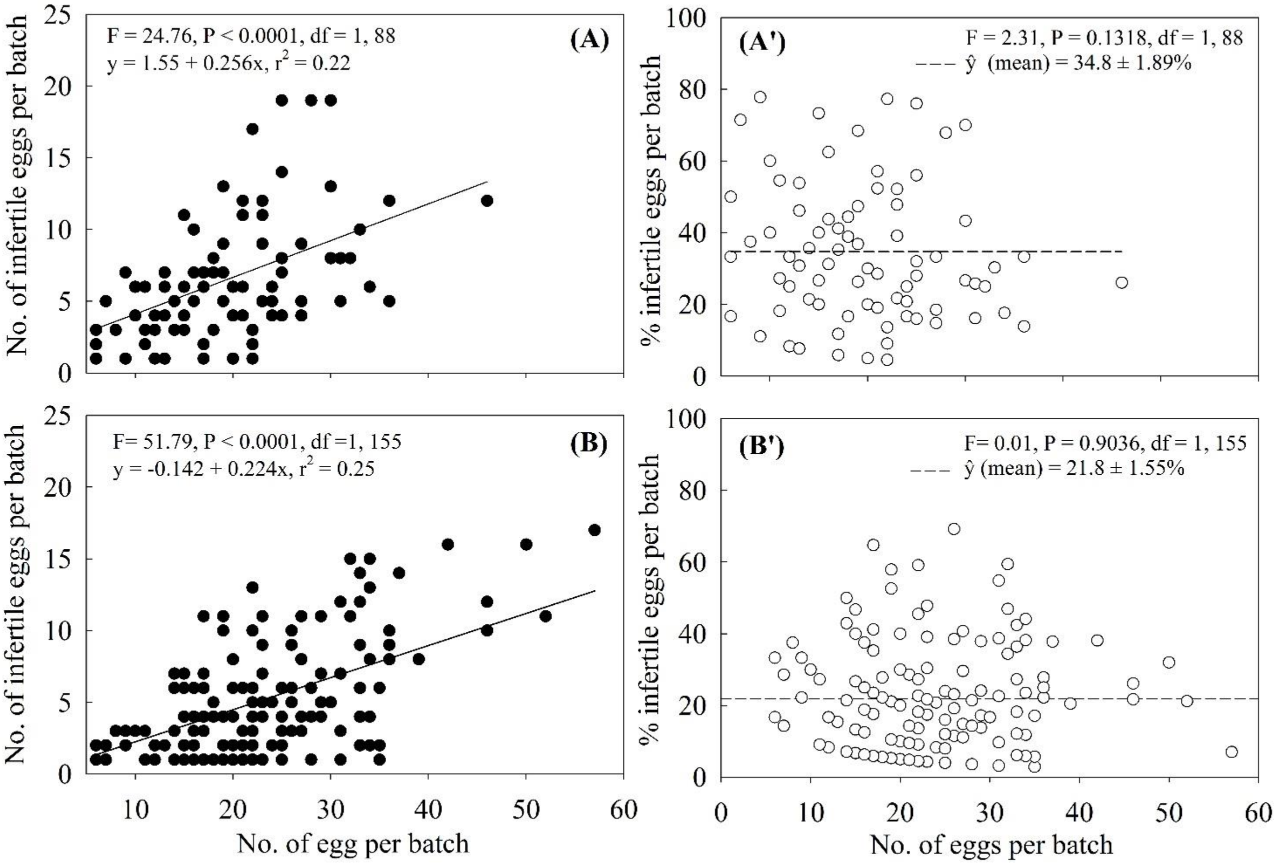

Figure 1. Number (A and B) or percentage (A' and B') of infertile eggs per egg batch for *Eriopis connexa* in egg batches randomly collected from the rearing colony (A and A') or from the selected 2nd and 3rd produced egg batches per female (B and B').

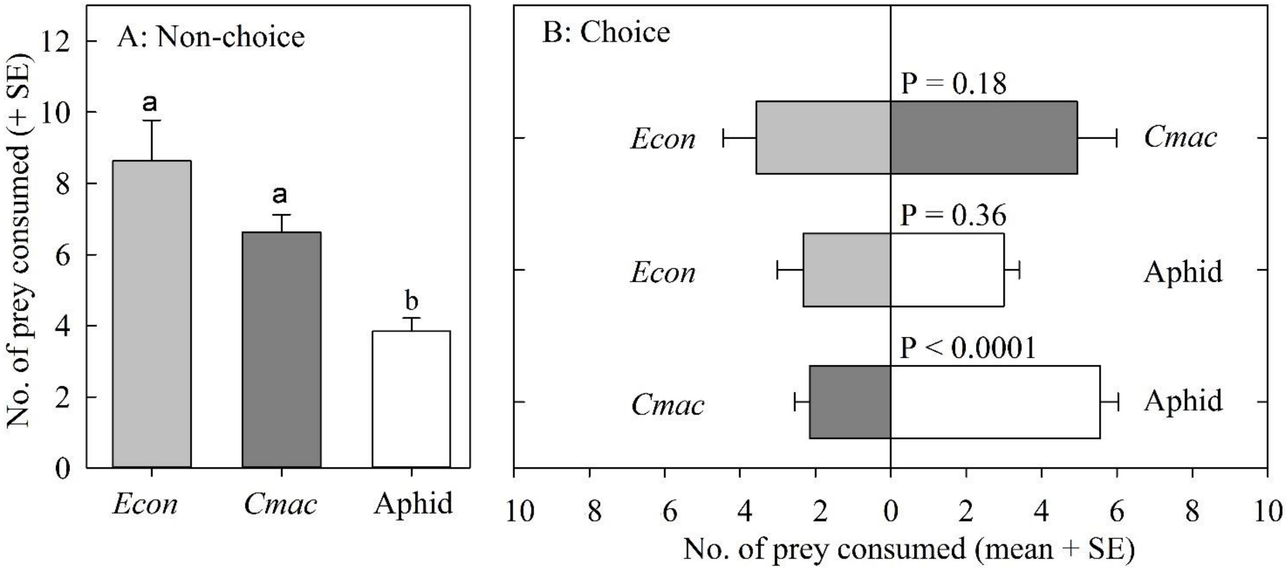

Figure 2. Predation by 3-day-old *Eriopis connexa* (*Econ*) larvae upon conspecific (*Econ*) eggs, heterospecific (*Coleomeguilla maculata*: *Cmac*) eggs, or turnip aphid (*Lipaphis pseudobrassicae*), the preferred extraguild prey in a non-choice test (A) or predation in a choice test comparing cannibalism vs. intraguild prey (*Econ* vs. *Cmac*), cannibalism vs. extraguild prey (*Econ* vs. aphid) or intraguild vs. extraguild prey (*Cmac* vs. aphid). In (A), different letters indicate statistical differences by Tukey HSD's test (α = 0.05), while p-values in (B) are based on Chi-square tests.

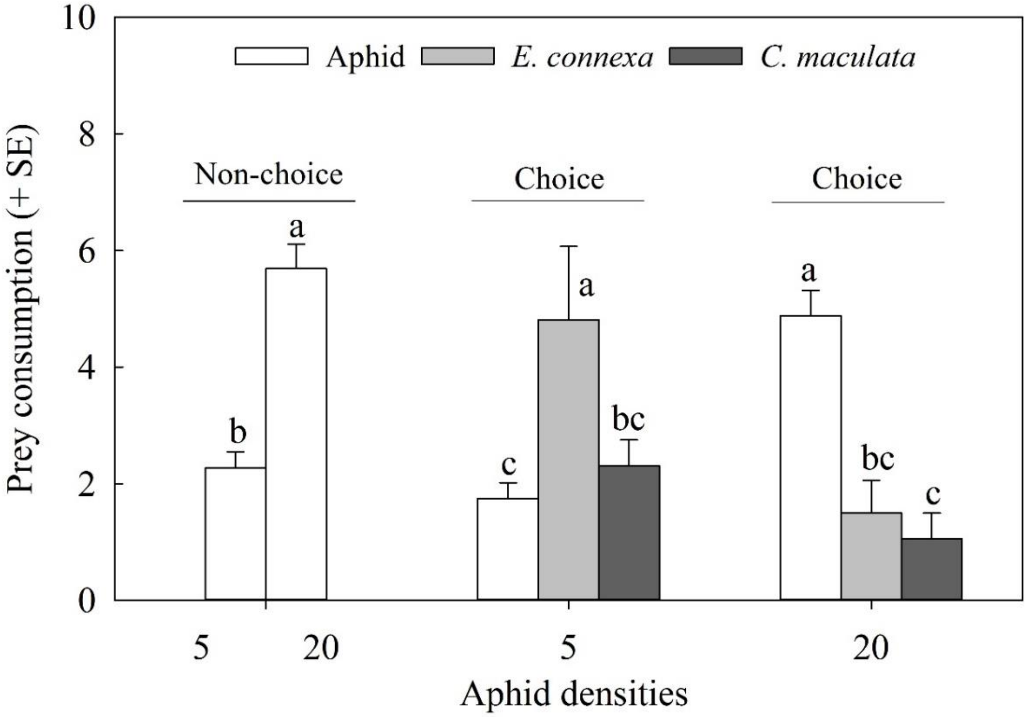

Figure 3. Predation by 3-day-old *Eriopis connexa* larvae upon the turnip aphid, *Lipaphis pseudobrassicae* at two densities and when simultaneous available 20 eggs of the conspecific (*E. connexa*), heterospecific (*Coleomegilla maculata*) species. Bars bearing different letters indicate statistical differences by pairwise (non-choice test) or multiple pairwise Chi-square tests (α = 0.05).

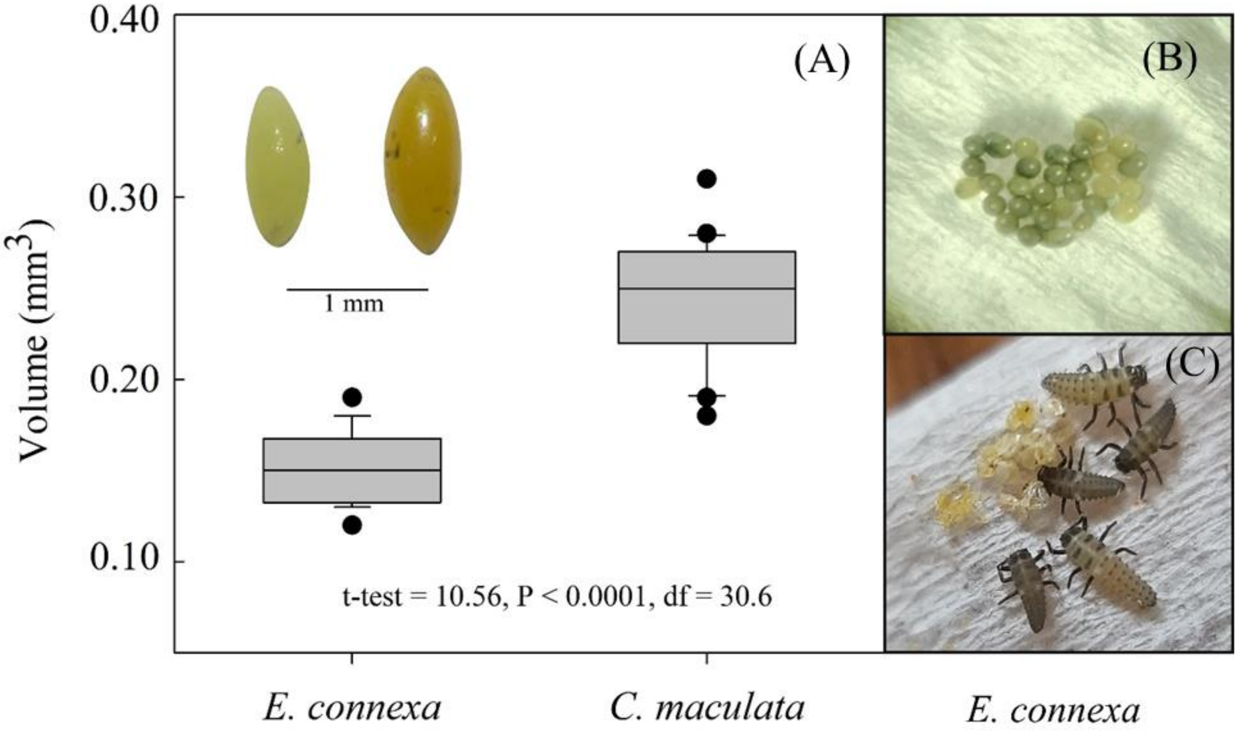

Figure 4. Estimated volume of *Eriopis connexa* and *Coleomegilla maculata* eggs (A), an *E. connexa* egg batch showing fertile (darker) and infertile (pale) eggs (B), and newly emerged larvae feeding on egg remains and infertile eggs (C).

CAPÍTULO 4

CONSIDERAÇÕES FINAIS

De acordo com os nossos resultados, é possível afirmar que a maturidade reprodutiva de *Eriopis connexa* (Germar) começa a se desenvolver aos três dias de idade para ambos os sexos. Contudo, machos e fêmeas só foram considerados completamente maduros sexualmente aos cinco dias de idade. O horário de maior atividade sexual durante a fotofase foi registrado na primeira hora de pareamento entre 7h e 8h da manhã, para indivíduos virgens sexualmente maduros, o que sugere que adultos virgens em idade reprodutiva estão propensos a copular tão logo encontrem um parceiro. O número médio de cópulas registrado foi de 6,1 cópulas por casal, com até 21 cópulas durante 12h de fotofase. A duração média de uma cópula é de 23,9 minutos, com variação de 5 a 90 minutos.

Machos de *E. connexa* caracterizados pelo fenótipo de suscetibilidade e resistência a piretroides, possuem as mesmas chances de acasalar com fêmeas suscetíveis, visto que as fêmeas não apresentaram preferência para acasalar, tampouco se mostraram refratárias ao fenótipo oposto após a cópula, independente de qual macho acasalou primeiro. Este resultado é importante para a conservação do fenótipo em campo, e para programas de liberação de indivíduos resistentes, os quais terão as mesmas chances de acasalar com fêmeas de ambos os fenótipos, gerando descendentes F1 resistentes desses acasalamentos, devido ao modo de herança da resistência.

A seleção pós-copulatória, caracterizada pela competição entre os espermatozoides de diferentes machos, ou por uma escolha das fêmeas é bastante estudada em joaninhas. Em *E. connexa*, nossos resultados fornecem fortes indícios de que a paternidade dos descendentes da mesma postura pode ser compartilhada por pelo menos dois machos distintos, o que indica a mistura de espermatozoides na espermateca.

A fertilidade em *E. connexa* também não é afetada pelo fenótipo resistente a piretroides. Fêmeas de ambas as populações, suscetível e resistente, depositaram ovos inférteis em proporções similares e, nem a adição de ovos conspecíficos ou da presa intraguilda, *Coleomegilla maculata* (DeGeer), influenciaram a fertilidade de *E. connexa.* Contudo, a fecundidade foi aumentada tanto pelo canibalismo, quanto pela predação intraguilda de ovos. Além disso, na presença da presa extraguilda (pulgão das brássicas) em baixa densidade, larvas de *E. connexa* preferiram o canibalismo de ovos à presa intraguilda (ovos de *C. maculata*). Porém ambos, o canibalismo e a predação intraguilda, foram igualmente reduzidos em alta densidade da presa extraguilda. Este é um resultado interessante para a coexistência destes predadores, visto que interações de *E. connexa* e outros predadores afidófagos são esperadas por ocorrerem no mesmo habitat e explorarem a mesma presa, características estimuladoras para a predação intraguilda. Contudo, em campo, há outros fatores que interferem nessas interações, como horário de forrageamento, particionamento de nicho, capacidade dispersão na planta, entre outros, e estudos de campo ou semi-campo podem ajudar a esclarecer estas questões.

Fêmeas de diversas espécies, incluindo muitos coccinelídeos, depositam ovos inférteis para a nutrição delas próprias, e em especial dos descendentes recém eclodidos. A hipótese deste investimento maternal na produção de ovos inférteis em joaninhas predadoras é de favorecer a sobrevivência das larvas recém eclodidas, as quais são frequentemente submetidas à condição de escassez de presa pela sua baixa dispersão e eclosão em grupo, aumentando o risco de canibalismo entre as larvas. As condições de escassez de presa para larvas e adultos, canibalismo e predação intraguilda de ovos e o fenótipo de resistência não fizeram com que fêmeas de *E. connexa* produzissem mais ovos inférteis. Por outro lado, a produção de ovos inférteis teve a mesma proporção, aumentando o número de ovos inférteis com o aumento de ovos por postura, consequentemente, aumentando a disponibilidade de ovos inférteis para o maior número de larvas eclodidas. O canibalismo de ovos provou ser capaz de permitir o desenvolvimento, porém com consequências para a reprodução e, portanto, para sua conservação em campo. Nesse contexto, estudos morfofisiológicos poderiam esclarecer quais os efeitos diretos da escassez alimentar no sistema reprodutivo de machos e fêmeas de *E. connexa*.

Portanto, nossos resultados irão contribuir para o melhor entendimento da reprodução em coccinelídeos, particularmente para *E. connexa*. Além disso, este estudo compreende aspectos fundamentais para sua utilização em pesquisas científicas e multiplicação para liberações, além de demonstrar através da experimentação, como fatores ambientais como a escassez de presas e interações intra- e interespecíficas poderiam afetar a reprodução neste predador. Ademais, estudos futuros poderão esclarecer questões concernentes à reprodução em *E. connexa*.

SUPLEMENTARY MATERIAL

Table 1S. Numbers of infertile eggs estimated as the difference between egg hatch (%) as published in different studies run with the resistant and susceptible studied *Eriopis connexa* phenotypes, and undefined phenotype.

Prey used and treatments	Egg infertility (100-% hatching)	References
E. connexa susceptible phenotype (Sus)		
Ephestia kuehniella (*Ek*) eggs F19	100-51.3 (48.7)	Santos *et al.* 2016
Ek eggs F24	100-43.9 (56.1)	Santos *et al.* 2016
Ek eggs F29	100-39.3 (60.7)	Lira *et al.* 2016
Ek eggs field collected (F1)	100-67.3 (32.7)	Lira *et al.* 2016
Ek eggs F78	100-31.9 (68.1)	Rodrigues *et al.* 2020
Ek eggs F94	100-51.0 (49.0)	Nascimento *et al.* 2021
Ek eggs + *P. xylostella* (*Px*) larvae	100-56.0 (44.0)	Nascimento *et al.* 2021
Lipaphis pseudobrassicae (*Lp*)	100-57.1 (42.9)	Nascimento *et al.* 2021
Alternated prey *Ek-Px-Lp*	100-52.4 (47.6)	Nascimento *et al.* 2021
Ek eggs	100-32.2 (67.8)	Ferreira *et al.* 2013
Ek eggs & paired once with virgin male	100-82.2 (17.8)	Colares *et al.* 2015
Ek eggs & paired continuously	100-68.7 (31.3)	Colares *et al.* 2015
Ek eggs & paired w/different virgin males	100-77.8 (22.2)	Colares *et al.* 2015
Ek eggs & paired w/different mated males	100-68.6 (31.4)	Colares *et al.* 2015
Ek eggs & untreated	100-65.1 (34.9)	Costa *et al.* 2020
Ek eggs & treated 0.08 g i.a./L spinetoram	100-70.5 (29.5)	Costa *et al.* 2020
Ek eggs & treated 0.12 g i.a./L spinetoram	100-65.2 (30.8)	Costa *et al.* 2020
Ek eggs & treated 0.24 g i.a./L spinetoram	100-56.2 (43,8)	Costa *et al.* 2020
Average (± 95% CI)	**42.2 ± 6.77**	

Table 1S. Cont.

E. connexa resistant phenotype (Res)		
Ek eggs & untreated	100-47.9 (52.1)	Santos *et al.* 2016b
Ek eggs & recovered from kd 24h	100-47.8 (52.2)	Santos *et al.* 2016b
Ek eggs & untreated	100-33.0 (67.0)	Santos *et al.* 2016b
Ek eggs & recovered from kd 24h	100-41.2 (58.8)	Santos *et al.* 2016a
Ek eggs & recovered from kd 48h	100-33.0 (67.0)	Santos *et al.* 2016a
Ek eggs & recovered from kd 72h	100-28.0 (72.0)	Santos *et al.* 2016a
Ek eggs F40	100-44.8 (55.2)	Lira *et al.* 2016
Ek eggs & from crossing Sus-F38 x Res-F40	100-75.9 (24.1)	Lira *et al.* 2016
Ek eggs F45	100-71.9 (28.1)	Lira *et al.* 2016
Ek eggs & from crossing Sus-F1 x Res-F45	100-75.9 (24.1)	Lira *et al.* 2016
Ek eggs & selected F78	100-39.2 (60.8)	Rodrigues *et al.* EEA
Ek eggs & unselected for 6 generations	100-43.0 (57.0)	Rodrigues *et al.* EEA
Ek eggs F9	100-24.6 (75.4)	Ferreira *et al.* 2013
Ek eggs & treated with dose 0.05 mg a.i./L lambda-cyhalothrin	100-31.4 (68.6)	Ferreira *et al.* 2013
Ek eggs & treated with dose 0.10 mg a.i./L lambda-cyhalothrin	100-33.3 (66.7)	Ferreira *et al.* 2013
Ek eggs & treated with dose 0.25 mg a.i./L lambda-cyhalothrin	100-43.5 (56.5)	Ferreira *et al*. 2013
Ek eggs & untreated	100-76.1 (23.9)	Costa *et al.* 2020
Ek eggs & treated 0.08 g i.a./L spinetoram	100-65.8 (34.2)	Costa *et al.* 2020
Ek eggs & treated 0.12 g i.a./L spinetoram	100-69.8 (30.2)	Costa *et al.* 2020
Ek eggs & treated 0.24 g i.a./L spinetoram	100-60.2 (39.8)	Costa *et al.* 2020
Average (± 95% CI)	**50.7 ± 7.71**	
***E. connexa* undefined phenotype**		
Fed *Diatraea saccharalis* eggs	100- 36.4 (63.6)	Silva *et al*. 2010a
Fed *Schizaphis graminum*	100-45.1 (54.9)	Silva *et al.* 2010b
Fed *Ek* eggs	100-84.5 (15.5)	Zazyckia *et al*. 2015
Fed *Myzus persicae* and *Toxoptera aurantii*	100-70.0 (30.0)	Gómez & Polanía 2009
Fed *Aphis gossypii* reared on cotton	100-69.1 (30.9)	Correa *et al.* 2014

BRS Rubi		
Fed *A. gossypii* reared on cotton BRS Safira	100-65.2 (34.8)	Correa *et al.* 2014
Fed *A. gossypii* reared on cotton BRS Verde	100-49.9 (50.1)	Correa *et al.* 2014
Fed *Drosophila melanogaster* larvae	100-62.8 (33.8)	Almeida *et al.* 2021
Fed *Ek* eggs	100-69.4 (30.6)	Almeida *et al.* 2021
Fed *Macrosiphum rosae*	100-58.7 (41.3)	Pereira 2017
Average (± 95% CI)	**38.5 ± 8.76**	

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