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TESE

Caracterização de *Trichopria* spp. e suas interações com *Drosophila melanogaster* e *Drosophila suzukii*

Alexandra Peter Krüger

Pelotas, 2022

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“*If you understand what you are doing, you are not learning anything.”*
Abraham Lincoln

Resumo

KRÜGER, Alexandra Peter. **Caracterização de *Trichopria* spp. e suas interações com *Drosophila melanogaster* e *Drosophila suzukii.*** 2022. 161f. Tese (Doutorado) – Programa de Pós-graduação em Fitossanidade, Universidade Federal de Pelotas, Pelotas, 2022.

Drosophila suzukii (Matsumura, 1931) (Diptera: Drosophilidae) é uma importante praga de pequenas frutas em diversos países ao redor do mundo, majoritariamente controlada com o uso de inseticidas. Porém, o uso de controle biológico, especialmente com parasitoides pupais, tem sido incentivado e estudado. Ainda, *D. suzukii* é uma espécie próxima de *Drosophila melanogaster* Meigen, 1830 (Diptera: Drosophilidae), inseto bem caracterizado geneticamente, permitindo o seu uso em estudos comparativos. *Trichopria* spp. (Hymenoptera: Diapriidae) é um parasitoide pupal com potencial de uso como agente de controle biológico de *D. suzukii.* Dessa forma, o objetivo desse trabalho foi caracterizar aspectos de *Trichopria drosophilae* Perkins, 1910 (Hymenoptera: Diapriidae) e *Trichopria anastrephae* Lima, 1940 (Hymenoptera: Diapriidae), bem como suas interações com *D. melanogaster* e *D. suzukii*, visando obter informações que suportem o uso deste parasitoide no controle biológico da praga. Para tanto, foram realizados estudos sobre o veneno e os teratócitos de *T. drosophilae*, além de estudos sobre as estratégias de acasalamento, efeitos da ausência hospedeira e da competição intraespecífica de *T. anastrephae.* Ainda, foi avaliada a capacidade deste parasitoide em buscar e parasitar pupas de *D. suzukii* presentes em morangos. Dentre as proteínas presentes no veneno de *T. drosophilae* estão apolipoforinas, enzimas proteolíticas, endonucleases e toxinas, que afetam genes Turandot, proteases, inibidores de proteases e P450 no hospedeiro. Teratócitos foram descobertos e descritos. Foi observada protandria e poligamia em *T. anastrephae*, e seus efeitos sobre o parasitismo e a produção de fêmeas foram descritos. Além disso, resultados de estudo de competição sugerem a ocorrência de superparasitismo, porém, este fenômeno não afetou a viabilidade da progênie, e a privação de hospedeiros por um período de até 7 dias não influenciou na capacidade de parasitismo das fêmeas. Fêmeas de *T. anastrephae* foram capazes de distinguir entre morangos infestados e não infestados por *D. suzukii* em laboratorio*,* e encontrar e parasitar pupas presentes em morangos em casa de vegetação, a ponto de diminuir a população da praga. Os resultados presentes neste trabalho incentivam o estudo e uso de *T. anastrephae* como agente de controle biológico de *D. suzukii.*

Palavras-chave: parasitoide pupal. Drosófila-da-asa-manchada. Estratégias reprodutivas. Teratócitos. Comportamento

Abstract

KRÜGER, Alexandra Peter. **Characterization of *Trichopria* spp. and its interactions with *Drosophila melanogaster* e *Drosophila suzukii*.** 2022. 161f. Tese (Doutorado) – Programa de Pós-Graduação em Fitossanidade. Universidade Federal de Pelotas, Pelotas, 2022.

Drosophila suzukii (Matsumura, 1931) (Diptera: Drosophilidae) is an important pest of soft skinned fruits in different countries around the world, mainly controlled with insecticides. However, the use of biological control, specially with pupal parasitoids, has been incentivated and studied. Yet, *D. suzukii* is a close species to *Drosophila melanogaster* Meigen,1830 (Diptera: Drosophilidae), which is well genetically characterized, allowing its use in comparative studies. *Trichopria* spp. (Hymenoptera: Diapriidae) is a pupal parasitoid with potential of being used as a biological control agent of *D. suzukii.* Thus, the goal of this work was to characterize aspects of *Trichopria drosophilae* Perkins, 1910 (Hymenoptera: Diapriidae) and *Trichopria anastrephae* Lima, 1940 (Hymenoptera: Diapriidae) as well as their interactions with *D. melanogaster* and *D. suzukii,* aiming to obtain more information to support the use of this parasitoid in the biological control of the pest. Therefore, studies were carried out on the venom and teratocytes of *T. drosophilae*, besides studies about mating strategies, host deprivation and intraspecific competition of *T. anastrephae.* Yet, the capacity of this parasitoid to search and parasite *D. suzukii* pupae in strawberry was evaluated. Among the proteins found in *T. drosophilae* venom are apolipophorins, proteolytic enzymes, endonucleases and toxins, that affect Turandot genes, proteases, protease inhibitors and P450 in the host. Teratocytes were discovered and broadly described. Protandry and polygamy was observed in *T. anastrephae,* and their effects on parasitism and female production was described. Moreover, results of competition suggest the occurrence of superparasitism, but this phenomenon did not affect the progenie viability, and host deprivation for a period of up to 7 days did not influence the parasitism capacity of females. *T. anastrephae* females were able to distinguish between *D. suzukii*-infested and non-infested strawberries in laboratory, and to find and parasitize pupae present in strawberries in greenhouse, at the point of lower the pest population. Results present in this work incentive the study and use of *T. anastrephae* as biological control agent of *D. suzukii.*

Keywords: pupal parasitoid. Spotted-wing Drosophila. Reproductive strategies. Teratocytes. Behavior.

Lista de Figuras

Artigo 1

Artigo 2

Artigo 3

Artigo 4

Lista de Tabelas

Artigo 1

Artigo 2

Artigo 3

Artigo 4

Sumário

1- Introdução

Atualmente, o Brasil é o terceiro maior produtor mundial de frutas, com uma produção em crescente ascenção, tendo atingido aproximadamente 44 milhões de toneladas no ano de 2020, sendo que a exportação de frutas gerou para o país uma receita de 875 milhões de dólares (ANUÁRIO BRASILEIRO DE HORTI & FRUTI, 2021). Um dos principais limitantes para a produção e exportação de frutas é a ocorrência de insetos-praga, que podem causar danos diretos e indiretos à fruticultura.

Dentre os principais insetos-praga, encontram-se espécies invasoras que se estabelecem em determinado local/região, e causam grandes perdas na produção (ASPLEN et al., 2015). Um exemplo de espécie invasora que tem causado danos na fruticultura, principalmente em frutos de epicarpo delgado, é a *Drosophila suzukii* (Matsumura, 1831) (Diptera: Drosophilidae). Esta espécie é nativa da Ásia, mas atualmente encontra-se amplamente disseminada na Europa e nas Américas do Sul e do Norte (WALSH et al., 2011; CALABRIA et al., 2012; DEPRÁ et al., 2014), e mais recentemente tendo sido reportada também no continente africano (BOUGHDAD et al., 2020).

No Brasil, a presença de *D. suzukii* foi reportada pela primeira vez na região sul do país em 2013 (DEPRÁ et al., 2014), e apenas um ano após o seu primeiro registro, a espécie foi associada a ocorrência de perdas de até 30% em cultivos de morango no noroeste do Rio Grande do Sul (SANTOS, 2014). Dois anos após este relato, produtores de Minas Gerais, o estado com maior produção de morangos no Brasil, também relataram a ocorrência de altos níveis de perdas devido a ocorrência de *D. suzukii* (ANDREAZZA et al., 2016). A capacidade de *D. suzukii* de causar danos em frutos com tegumento íntegro, diferentemente da

maioria das espécies da família Drosophilidae, é devido ao ovipositor esclerotizado e serrilhado, que permite as fêmeas ovipositarem no interior dos frutos, onde então as larvas se desenvolvem (SCHLESENER et al., 2014).

Em países da Europa e da América do Norte, a principal forma de manejo de *D. suzukii* é através do controle químico, com produtos organofosforados, piretroides e espinosinas (BRUCK et al., 2011; BURRACK et al., 2015). No Brasil apenas um produto do grupo das espinosinas (espinetoram) está registrado para o manejo dessa espécie nos cultivos de framboesa, mirtilo e uva (AGROFIT, 2020). Porém, devido ao ciclo de vida curto da *D. suzukii* e o controle residual limitado promovido pelos inseticidas, estes produtos necessitam de aplicação frequente para manter o nível populacional do inseto baixo (RENKEMA et al., 2016). Além dos riscos para o ambiente e para a saúde do consumidor e do aplicador, causados pela aplicação frequente dos inseticidas, também existe o risco do desenvolvimento da resistência dos insetos aos inseticidas (GRESS; ZALOM, 2019).

O controle biológico é uma importante estratégia alternativa ao uso de inseticidas químicos. No caso de *D. suzukii*¸ os principais agentes de controle biológico estudados são os parasitoides, porém, devido ao seu poderoso sistema imune, poucas espécies de parasitoides conseguem completar o desenvolvimento no interior desta praga (KACSOH; SCHLENKE, 2012). Comparado aos parasitoides larvais, os parasitoides pupais parecem ter mais facilidade em completar o desenvolvimento em pupas de *D. suzukii*, com destaque para as espécies mais estudadas: *Pachycrepoideus vindemmiae* (Rondani, 1875) (Hymenoptera: Pteromalidae), *Trichopria drosophilae* Perkins, 1910 (Hymenoptera: Diapriidae) e *Trichopria anastrephae* Lima, 1940 (Hymenoptera: Diapriidae) (ROSSI-STACCONI et al., 2015; VIEIRA et al. 2020a, GARCIA et al., 2022). No caso de *P. vindemmiae,* este sucesso é atribuído ao veneno injetado pela fêmea do parasitoide, no momento do parasitismo, que é capaz de alterar a fisiologia do hospedeiro (YANG et al. 2019, 2020).

No Brasil, duas espécies de parasitoides pupais já foram encontradas no ambiente, associadas a *D. suzukii*: *P. vindemmiae* e *T. anastrephae* (WOLLMANN et al., 2016; ANDREAZZA et al., 2017, GARCIA et al., 2022). Porém, *P. vindemmiae* tem como desvantagem o fato de ser um hiperparasitoide de Hymentoptera benéficos (WANG; MESSING, 2004), enquanto que *T.*

anastrephae tem sido considerado o parasitoide com maior potencial no controle de *D. suzukii* na América Latina (GARCIA 2020, GARCIA et al., 2022).

Devido a este potencial, recentemente, foram realizados estudos que forneceram informações importantes para a viabilização de criações massais de *T. anastrephae* em pupários de *D. suzukii* (KRÜGER et al., 2019; VIEIRA et al., 2020a), e sobre efeitos de diferentes temperaturas sobre o desenvolvimento e tabela de vida do parasitoide (VIEIRA et al., 2020b). Além disso, também foi demonstrado a competição deste parasitoide com *P. vindemmiae* (OLIVEIRA et al., 2021), a compatibilidade de *T. anastrephae* com inseticidas (SCHLESENER et al., 2019; MORAIS et al., 2022) e a habilidade do parasitoide em discriminar frutos de mirtilo infestados e não infestados por *D. suzukii* (DE LA VEGA et al., 2021).

Visto o potencial do parasitoide pupal *T. anastrephae* para o controle biológico de *D. suzukii* na América Latina, o presente trabalho teve como objetivo caracterizar *T. drosophilae* e *T. anastrephae,* bem como suas interações entre este parasitoide com o hospedeiro *D. suzukii*, a fim de obter mais informações para fundamentar o controle biológico da praga. Para atingir este objetivo, o presente trabalho foi dividido em quatro partes principais: na primeira parte, devido a disponibilidade de dados transcripcionais e genômicos, foram desenvolvidos ensaios utilizando como modelos o parasitoide *T. drosophilae* e o hospedeiro *D. melanogaster*, para verificar os fatores envolvidos no sucesso do desenvolvimento do parasitoide no interior do hospedeiro. Na segunda parte, foram realizados uma sequência de estudos para entender as estratégias de acasalamento empregadas por *T. anastrephae* para se multiplicar em *D. suzukii.* Na terceira parte, foram realizados experimentos para avaliar os efeitos da ausência hospedeira e da competição intraespecífica no parasitismo de *T. anastrephae*. Finalmente, na quarta parte foram realizados experimentos para verificar a capacidade de *T. anastrephae* buscar e parasitar pupas de *D. suzukii* presentes em frutas de morango, em laboratório e casa de vegetação.

Artigo 1 – Journal of Insect Physiology

2- Artigo 1 - Factors involved in the parasitism success of *Trichopria drosophilae*

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Abstract

Hymenopteran parasitoids use strategies to overcome host immune defenses and ensure an amenable environment for their development. Among these strategies are venom and teratocytes. While venom is injected by some wasp females in the moment of the oviposition, teratocytes derive from a membrane that encloses the embryo of some species of parasitoids. Hosts insects have also evolved a series of innate defense responses. In this study, we described both the parasitism strategy of the pupal parasitoid *T. drosophilae,* as well as its hosts, *D. melanogaster,* immediate immune response. We conducted a venom proteome of *T. drosophilae,* and the immune response via differential expression of it's host when naturally parasitized and injected with venom. In addition, we discovered and conducted a transcriptomic profile of *T. drosophilae*'s teratocytes. We found that the venom of this species is composed of a variety of proteins including apolipophorin, proteolytic enzymes, endonucleases and toxins. Injection of venom in *D. melanogaster* pupa resulted in the up-regulation of 40 genes, including several Turandot genes, proteases and protease inhibitors, and the down-regulation of 6 genes, including P450. Newly discovered teratocytes originate from serosa cells at the moment of egg eclosion, grow in size after being released in the host hemocele, and disappear 7 days after parasitism. Teratocytes mainly express antimicrobial peptides, but also express proteases and lipoproteins. Results presented here suggest that the success of *T. drosophilae* parasitism and development within its host is associated with the venom injected by the female and the teratocytes derived from embryonic cells.

Keywords: venom, teratocytes, parasitism, proteins

Introduction

Hymenopteran parasitoids greatly depend on their host for larval development. Thus, parasitoids have evolved a series of strategies to overcome host immune defenses and maintain a supportive environment to successfully complete their development (Andrew et al., 2006; Carton et al., 2008; Schmidt et al., 2001; Shelby et al., 2000). Among these strategies are host immunosuppression, developmental arrest, and metabolic changes for parasitoid development (Beckage and Buron, 1993; Schmidt et al., 2001). In order to overcome host defenses and make the host habitable for her offspring's development, female parasitoids often inject a variety of different factors along with the egg. Among them are polydnaviruses, virus-like particles, ovarian proteins and venoms. Additionally, some species of parasitoids also generate extra-embryonic cells, termed teratocytes, which are released during larva hatching inside of the host (Beckage and Gelman, 2004; Pennacchio and Strand, 2006, Vinson et al., 1994).

Venom is produced by female parasitoids in a part of their reproductive tract called venom gland (Poirie et al., 2014), and is composed by a complex mixture of molecules that vary according to the species, and has a variety of functions, from permanently paralyzing the host to regulating the host physiology (Asgari and Rivers, 2011; Cusumano et al., 2018; Morea and Asgari, 2015; Poirie et al., 2009; Poirie et al., 2014). Venom may also regulate the host immune response by inducing developmental alterations and display antimicrobial activity (Colinet et al., 2009; Shen et al., 2009; Zhu et al., 2009). Some species of parasitoids also produce polydnavirus and/or virus-like particles in specific ovarian tissue called the calyx or in the venom apparatus that may synergize with the effects of venom on host regulation, or overlap its functions (Asgari and Rivers, 2011; Doremus et al., 2013).

Teratocytes are cells originating from the amnion or the serosal cell membrane enclosing the parasitoid embryo in some hymenopteran species (Strand, 2014). Upon hatching, the membrane dissociates into individual cells that develop autonomously in the host hemolymph along with the parasitoid larva (Andrew et al., 2006). The number of cells is species-dependent, varying from 12 to 800 cells per parasitoid egg (Rouleux-Bonnin et al., 1999). Teratocytes have been described only in endoparasitoid species of Aphelinidae, Braconidae, Mymaridae, Plastygasteridae, Scelionidae and Trichogrammatidae (Dahlman and Vinson, 1993; Hotta et al., 2001; Basio and Kim, 2005) and some teratocyte-like cells were recorded in Ichneumonidae (Roulex-Bonnin et al., 1999). As the parasitoid larva develop, teratocytes usually increase in size, and undergo cytological changes such as increase in the number of microvilli and vesicles (Tanaka and Wago, 1990). Most studies show that the nuclear diameter and ploidy may increase without the teratocytes dividing after dissociation (Strand and Wong, 1991; Hotta et al., 2001). However, one study demonstrated an increase in teratocyte number, likely by teratocyte division (Mancini et al. 2016) in Encarsia pergandiella. While the precise role of teratocytes remains unclear, they are considered to have multiple physiological functions to facilitate parasitoid larva survival and growth. They are involved in parasitoid larval nutrient uptake (Sluss, 1968; Strand et al., 1986), host immune depression (Vinson, 1970; Kitano et al., 1990; Tanaka and Wago, 1990), host developmental arrest (Zhang et al., 1992) and/or repression of competing parasitoids' development (Collier et al., 2002; Pedata et al., 2002).

In response to parasitism, host insects have evolved multiple innate defense responses (Hoffmann, 1995). Once the structural barrier of defense that protects the hemocoel from invasion is broken, by an injury or an infection, the insect rapidly activates proteolytic cascades that lead to blood coagulation and melanization (Gregorio et al., 2002). Then, a cellular immunity response is mounted, mainly mediated by different hemocyte types, and

after the host recognizes the parasitoid/pathogen as non-self, they encapsulate or phagocytose the invader (Samakovlis et al., 1990; Yang et al., 2019). And during a systemic infection a number of inducible effector molecules that are secreted into the hemolymph (Tzou et al., 2002).

Trichopria drosophilae Perkins (Hymenoptera: Diapriidae) is a pupal endoparasitoid of Drosophilidae species, and recently has been intensively studied due to its potential as biological agent of against *Drosophila suzukii* Matsumura (Diptera: Drosophilidae), a well-known invasive pest (Tait et al., 2021, Garcia et al. 2022). Even though *D. suzukii* larvae shows a high hemocyte load that gives resistance against larval parasitoids (Kacsoh and Schlenke, 2012), *T. drosophilae* is able to overcome any immune response present in *D. suzukii* pupae, and shows high fecundity, high host specificity, and high parasitization rates (Mazzetto et al., 2016; Wang et al., 2016; Kaçar et al., 2017). Moreover, when released in the field, *T. drosophilae* is able to decrease *D. suzukii* population (Rossi-Stacconi et al., 2018; Rossi-Stacconi et al., 2019; Gonzales-Cabrera et al., 2019). However, although it has been extensively studied, there is no study describing the host regulatory factors that allow the successful development of this parasitoid in its hosts, such as venom and teratocytes. In fact, to the best of our knowledge, teratocytes have not been described in any species of Diapriidae. In this paper, we will examine broadly how *T. drosophilae* parasitizes its host, *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), a relative of *D. suzukii*, and which immune responses it elicits when first attempting to overcome the host immune defenses. To do this, we generated a venom proteome of *T. drosophilae*, used differential transcriptomics to understand the effect of the parasitoid venom on a *D. melanogaster* host, and finally discovered and generated a transcriptomic profile of *T. drosophilae*'s teratocytes.

Methods

Insect rearing

Drosophila melanogaster was reared on cornmeal-molasses, yeast, and agar diet. *T. drosophilae* colony started from wasps collected in Winters, California in 2002, and were reared on *D. melanogaster* pupae. Both species were reared under controlled conditions (Temperature: 24 ± 1°C, Photophase: 12 h).

Venom proteome

Three hundred venom reservoirs of 2-5 d old *T. drosophilae* were dissected into a protease inhibitor cocktail, made by dissolving one tab of cOmplete™ Protease Inhibitor Cocktail in 1 mL of sterile phosphate buffered saline (PBS). Tissue was pelleted by centrifugation at 12000 g, and venom-containing supernatant was removed and stored in -80°C until use. The resulting 17 ug of protein were run out on a polyacrylamide gel and stained with Coomassie Blue. Seven partitions were made based on size and brightness of the bands (Figure S1). Each partition was analyzed using Liquid Chromatography with tandem mass spectrometry (LC-MS-MS). The resulting fragments were identified using an assembled transcriptome of *T. drosophilae* (Zhou et al., 2019). Proteins with more than ten unique hits were identified as venom proteins.

Pupal injection with venom and transcriptome samples

Pilot experiments were conducted by injecting diluted venom into pupae and approximating the number of venom reservoirs per uL of solution (Table S1). From these, 2.5 venom reservoirs per microliter showed the phenotypic effect of slowed physiological development and no adult emergence, and were used to inject *D. melanogaster* pupae for transcriptomic analysis. For the transcriptome four treatments were prepared: 1. Natural parasitism of early *D. melanogaster* pupae (<24 h old), where early *D. melanogaster* pupae

were mounted on a petri dish and 2-5 d old *T. drosophilae* females were allowed to singly parasitize each pupa, 2. Unparasitized early *D. melanogaster* pupae, which were mounted on the petri dish, but not offered to *T. drosophilae* wasps, 3. Pupae were injected with 20nL of 2.5 venom gland/uL PBS (0.05 venom reservoir/pupae), 4. Sterile PBS injected pupae, which were pupae injected with 20 nL of sterile PBS. Pupae from the different treatments were flash-frozen 4 hours after parasitism/injection. Five individual pupae were pooled from each treatment and extracted using Trizol. Six samples from each treatment were sequenced with 150bp paired-end illumina HISeq. The resulting data was trimmed using Trimmomatic (version 0.39; settings (SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25). Using Pertea et al. (2016) as a guide, the trimmed reads were mapped to a reference *D. melanogaster* genome (BDGP6) using HISAT and indexed with StringTie. The differential expression analysis was performed in R using the BallGown module. Natural infection treatment was compared to the no injection control, and the venom injected treatment was compared to the PBS injected control.

Teratocyte development

Trichopria drosophilae were allowed to singly parasitize *D. melanogaster* pupae (<24 h old). Soon after parasitism and every 3h until eclosion, pupae were dissected and *T. drosophilae* embryo was collected. The embryos were fixed in 4% PBS-buffered paraformaldehyde for 20 minutes and washed for 3 minutes each with ethanol in increasing ethanol concentrations (50, 80, and 96% ethanol). Then, the samples were incubated in DAPI (10 mg/ml) for 5 minutes. The samples were imaged using Olympus BX53-f microscope with 10 x oculars connected to an Olympus DP74 color CMOS camera.

Teratocyte growth

T. drosophilae was allowed to singly parasitize *D. melanogaster* pupae (<24 h old), every 24 h after parasitism, five pupae were dissected on a glass slide, and number of teratocytes was counted and their areas were measured. In addition, three pupae were dissected and DAPI stained for counting and measuring the area of their nuclei.

Teratocyte transcriptomic profile

Individual *D. melanogaster* pupae (<24 h old) were allowed to be singly parasitized by *T. drosophilae* females. Four days after parasitism, 10 pupae/sample were dissected, parasitoid larvae were removed and the remaining slurry containing teratocytes were extracted for RNA using Trizol. Each of the three samples was treated with DNAse (Turbo DNAse kit), and sequenced with 150bp paired end Illumina HISeq. The resulting sequences were trimmed using the Trinity built-in version of Trimmomatic (version 0.39) with the default settings (SLIDINGWINDOW:4:5 LEADING:5 TRAILING:5 MINLEN:25). The trimmed sequences were denovo assembled using Trinity (version 2.8.4) into "transfragments". To separate transfragments belonging to *T. drosophilae* teratocytes from those originating from *D. melanogaster* pupae, all transfragments were filtered based on their blastx and DC-megablast results (against the NCBI nt and nr databases), and their percentage of coverage and identity against *D. melanogaster* genome, a reference *T. drosophilae* transcriptome, and other Hymenoptera genomes (Figure S2). Filtered transfragments were also annotated using the Trinotate pipeline (https://trinotate.github.io), (v 3.0.1) which uses different reference databases including SwissProt, NCBI, EggNOG and KEGG. Using Salmon (Patro et al. 2017), we normalized and estimated gene expression for each sample. The TPM (transcripts per million) were calculated for each teratocyte sample and the expressed genes were

organized in rank order according to their average TPM from each of the three biological replicates. GO-term plots were made for each log order of average TPM.

Results and discussion

Venom profile

According to proteome analysis, 545 proteins were found in the venom, with the number of hits to the *T. drosophilae* transcriptome varying from 845 to 1 hit (Table S2). The venom is quite varied, containing proteases, endonucleases, toxins, and proteins that could make microstructures. Among the 24 proteins over 100 unique hits, including apolipophorin with signal peptide, fatty acid synthetase, myosin heavy chain, proteolytic enzymes (4 aminopeptidases and neprilysin, the most abundant protein was an aminopeptidase), toxins (2 toxins with signal peptides), endonucleases [3 nucleases – with homology to *Nasonia vitripennis* (Walker) (Hymenoptera: Pteromalidae))], 2 proteins with lots of disorder domains that have the potential to form a membraneless organelle and 6 proteins that were most similar to a set of protein-coding genes in *Leptopilina heterotoma* (Thomson) (Hymenoptera: Figitidae), a generalist larval parasitoid of Drosophila flies.

The proteolytic enzymes, aminopeptidases and neprilysin, were also found in the venom of another pupal parasitoid of Drosophila flies, *Pachycrepoideus vindemmiae* Rondania (Hymenoptera: Pteromalidae) (Yang et al., 2019). Peptidases are components involved in the hydrolysis of host peptides and provide essential amino acids required for the development of the parasitoid larva (Yang et al., 2019). On the other hand, neprilysin is known to cleave proteins and potentially modulates the host's immune responses (Sitnik et al., 2014; Yang et al., 2019). Endonucleases were previously reported in the venom of the pupal parasitoids *N. vitripennis* and *P. vindemmiae* (De Graaf et al., 2010; Yang et al., 2019).

Their role is speculated to be connected to RNA cleavage of the host to confront its defensive responses (Trummal et al., 2016). Apolipophorins are proteins commonly found in parasitoid wasp venoms (Dorémus et al., 2013), and are involved in lipid metabolism, as well as fatty acid synthetase (Song et al., 2008).

Host response to venom

When comparing unmanipulated host vs parasitized host, no genes were statistically different when correcting for the false discovery rate generated by multiple tests (p value was significant, but the q value was not; Figure 1a). However, when comparing venom vs PBS injected pupae, 46 genes were significantly up or downregulated (Figure 1b). These genes were largely the same as in venom injected and naturally infected pupae, but there was a larger response when injecting venom. This could be because the amount of venom injected was higher than the actual amount that the female injects when parasitizing.

The highest differentially up-regulated gene in venom-injected pupae is a long, non-coding RNA with no known molecular function. The second-highest is a protein-coding and only expressed during the pupal phase, but otherwise also unknown. For the venom injected pupae, Turandot genes (TotZ, TotF, TotA, TotB and TotC) were also up-regulated. Turandot genes are a family of distantly related genes secreted by the fat body in response to stress (Lemaitre and Hoffmann, 2007), that have been previously studied in response to environmental stress and bacterial challenges (Ekengren and Hultmark, 2001), but not in response to injection of a parasitoid venom. Although the exact functions of Turandot genes are unknown, the induction of genes of this family is thought to have a role in the protection against tissue damage (Ekengren and Hultmark, 2001).

The majority of the other up-regulated genes are proteases and protease inhibitors, including several proteins involved in serine endopeptidases and serine endopeptidase

inhibitors (serpins) activity. Serine proteases and serpins are involved in the activation of an important innate immunity response protein, the prophenoloxidase, which is involved in cellular and humoral defense (Colinet et al., 2009; Lu et al., 2014). Serpin has been shown to modulate melanization reactions in different species of insects after infection, including *D. melanogaster* (Reichhart et al., 2011; Shakeel et al., 2019). The induction of serine proteases and serpins by parasitoid venom might be to suppress the prophenoloxidase activation in the host defense system and avoid the process of hemocyte melanization (Shakeel et al., 2017; Merlin and Consoli, 2019).

Only six genes were significantly down-regulated, and most of them appear to be involved in pupal development. Notably, one is cytochrome P450, a detoxifying gene, that generally should be up-regulated, but perhaps this is a manipulation by the parasitoid venom. However, the down-regulation of P450 was previously shown in pupae of *Sarcophagha crassipalpis* Macquart (Diptera: Sarcophagidae) parasitized by *N. vitripennis* (Danneels et al., 2013), but further investigations are needed to understand the reasons for this down-regulation.

Teratocyte development and growth

The development of *T. drosophilidae*'s teratocytes within the embryo follows the development suggested by Strand (2014) for teratocytes derived from serosal cells (Figure 2). Briefly, meiosis of the oocyte nucleus results in the formation of the egg pronucleus surrounded by cytoplasm and polar bodies that will degenerate. Mitosis of the egg pronucleus produces additional nuclei that migrate to the periphery of the egg that will form the blastoderm cells. Blastoderm cells give origin to the embryo, serosa and amnion. At hatching, the wasp larva emerges, the serosa dissociates into teratocytes and the amnion degenerates (Strand, 2014).

On average, the number of teratocytes is variable between pupae, but not fluctuating a lot, with 50-70 teratocytes per pupae, two to seven days after parasitism (Figure 3). In our dataset, one pupa had approximately double the number of teratocytes 3 days after parasitism. We believe that this was a superparasitized pupae. After day 7, parasitoid larva reached the third instar, and teratocytes were no longer observed. As reviewed by Strand (2014), the number of teratocytes produced by a single egg of different wasp species vary from 20 to more than 1000, and this number usually declines while the size of teratocytes increases as the parasitoid larvae develop. The decline in the number of teratocytes may be due to wasp larvae consumption (Vinson 1980) or teratocyte death (Vinson, 1980; De Buron and Beckage, 1997).

After being released in the host, teratocytes of *T. drosophilae* grown in size but between days 3 and 4 (Figure 4 and Figure S3). On day 4, there were many teratocytes with two nuclei and lots of malformations (Figure 5 and Figure S4). The high number of teratocytes with two nuclei indicated DNA duplication between days 3 and 4. Polynucleate teratocytes were previously reported in the literature (Tremblay; Calvert 1971; Mancini et al. 2016), but evidence of cell division was only reported in *Encarsia pergandiella* Howard (Hymenoptera: Aphelinidae) (Mancini et al., 2016). Teratocytes of *T. drosophilae*, between days 4 and 5, seem to be attempting cytokinesis, but with variable degrees of success, and this is probably the reason why the number of teratocytes remain largely stable between day 4 and 5.

Teratocyte expression profile

The eight most highly expressed genes (over 10000 transcripts per million – TPM) were mitochondrial proteins and one gene encoding an antimicrobial peptide, interestingly classified as a gurmarin antifungal antimicrobial peptide. While further experimentation is

285 needed to define what the role of this antimicrobial peptide is, it clearly is a major part of the
286 teratocyte's function. In *Cotesia vestalis* (Kurdjumov) (Hymenoptera: Braconidae), their
287 teratocytes have been shown to produce antimicrobial peptides which inhibit bacterial growth
288 (Gao et al. 2016), and one of the major components of the proteins produced and exported by
289 teratocytes in *Microplitis demolitor* Wilkinson, (Hymenoptera: Braconidae) was also an
290 antimicrobial peptide (Burke and Strand 2014). Thus, it appears that one major function of
291 teratocytes is to be a source of defense molecules against pathogens while the wasp larva is
292 developing inside of the host, perhaps to compensate for the loss of defense mechanism by the
293 host itself.

294 In the next category, of genes with 1000-9999 TPM, we observed 111 genes in
295 different GO categories (Figure 6), but most of the genes were ribosomal proteins, which are
296 involved in translation, ribosomal structure and biogenesis, and genes categorized in post-
297 translational modification protein turnover, chaperones, including serine proteases and serine
298 protease inhibitors, and a gene with a weak homology to a pheromone binding protein. In this
299 category we also found actin, apolipoprotein and Ef-Tu. Apolipophorin was also observed in
300 the venom of *T. drosophilae*.

301 We found 701 genes 100- 999 TPM, and most of these genes in the category code for
302 translation and post-translation modification. However, more transport genes are represented
303 in this category as well (Figure 7), and some of these transport genes have signal peptides
304 where they normally might not: COPI (a coat protein usually involved in intracellular
305 transport) and cystonin (normally embedded in the lysosome to facilitate the transport of
306 cysteine molecules). In the next category (genes with 10-99 TPM) there are 4,874 genes, and
307 relative to the post-translational modification category, we observed a lot more intracellular
308 trafficking, secretion and vesicular transport genes, as well as transcription genes. Among the
309 genes over 0 TPM, we have once again an emphasis on the categories of translation,

transcription, replication, recombination and repair, cell cycle, signal transduction mechanism and intracellular trafficking, secretion and vesicle transport.

It appears that the teratocytes of *T. drosophilae* highly express antimicrobial peptides, various peptidases/other enzymes for digesting, transport, and express only very lowly genes for DNA replications, cell cycle and signal transduction.

Conclusion

This study described the *T. drosophilae*'s parasitism strategy, as well as the early defense and response mounted by the host pupa. *T. drosophilae* injects a variety of proteins along with its eggs into the host, including proteases, toxins, and endonucleases. The initial host response is to up-regulate many Toturant stress response genes as well as proteases and protease inhibitors. After approximately 15 hours, the larva hatches and the serosa releases teratocytes. The teratocytes produce antimicrobial peptides, many proteases and lipoproteins. These teratocytes might divide, but ultimately disappear once the wasp larva is old enough. While our study is descriptive, it was the first incidence to find teratocytes in the family Diapriidae, and it added another parasitoid venom profile to the literature. With *Drosophila suzukii* becoming a growing pest and *Trichopria* spp. a biological control agent of this species, this study can be a launching point for those studying this fascinating system.

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Figures

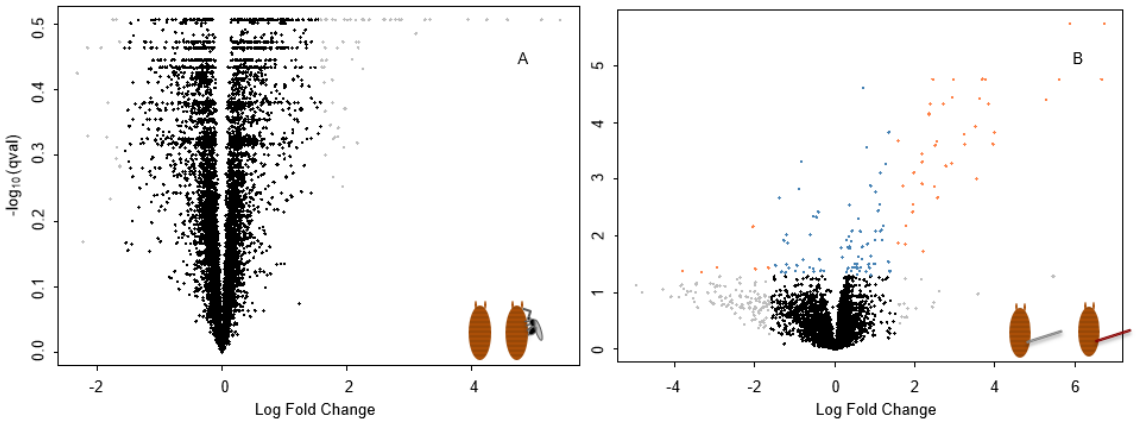


Figure 1. Volcano plots comparing up and down-regulated genes in (A) unmanipulated vs naturally parasitized pupae, and (B) PBS and Venom injected pupae.

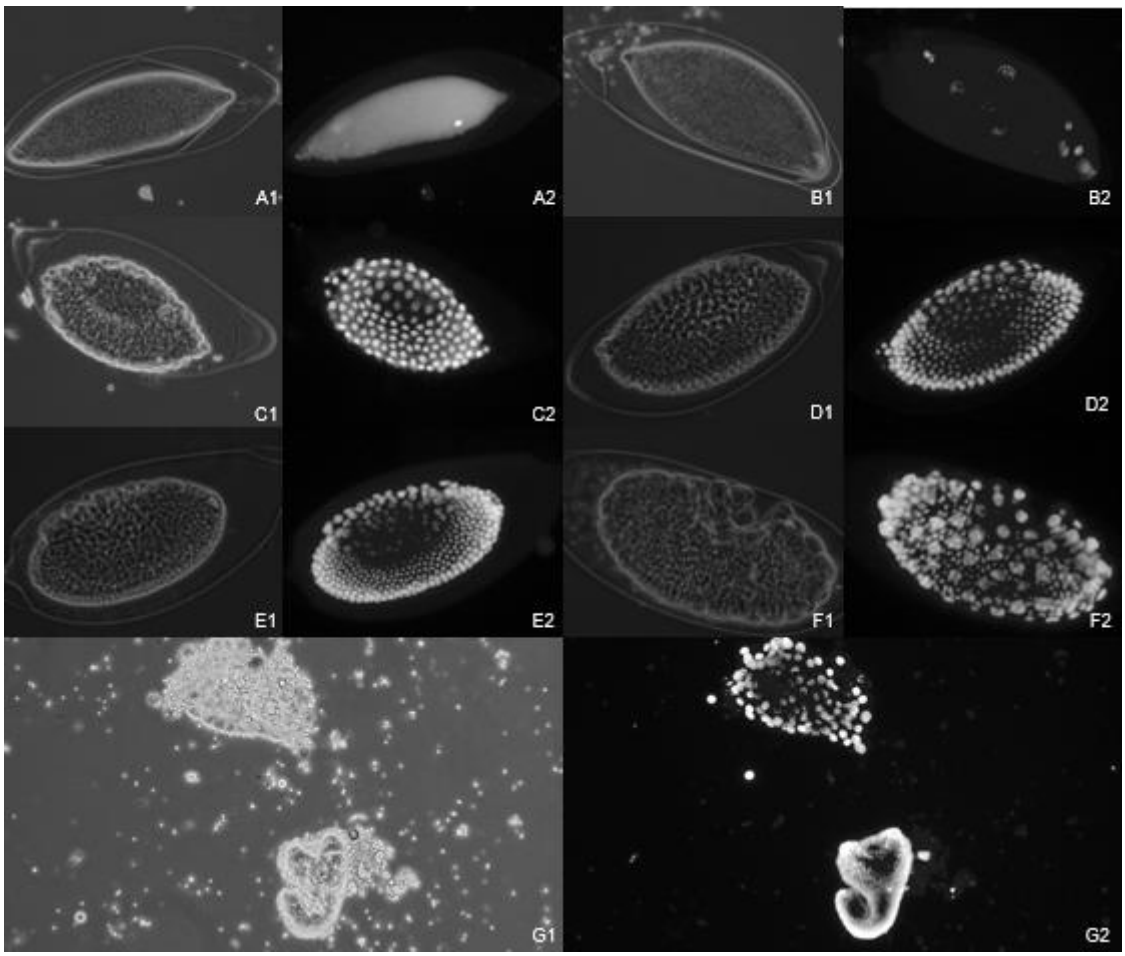


Figure 2. *Trichopria drosophilae* egg development at bright (-1) and DAPI (-2) field, at 0 (A), 3 (B), 6 (C), 9 (D), 12 (E) and 15 (F) hours after parasitism and eclosion, showing larva and serosal cells prior to dissociate into teratocytes (G)

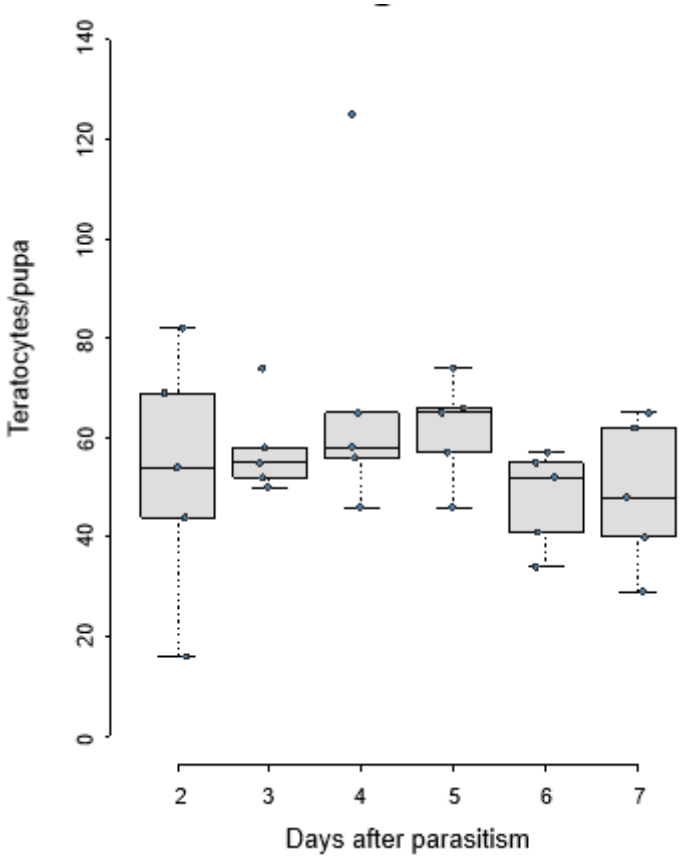


Figure 3. Number of teratocytes of *Trichopria drosophilae* per pupae of *Drosophila melanogaster*.

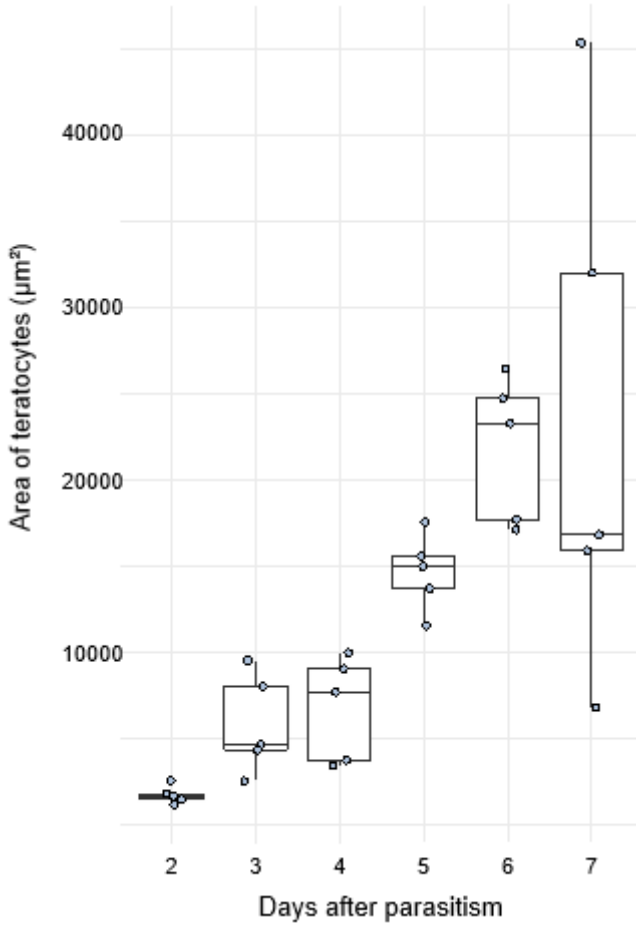
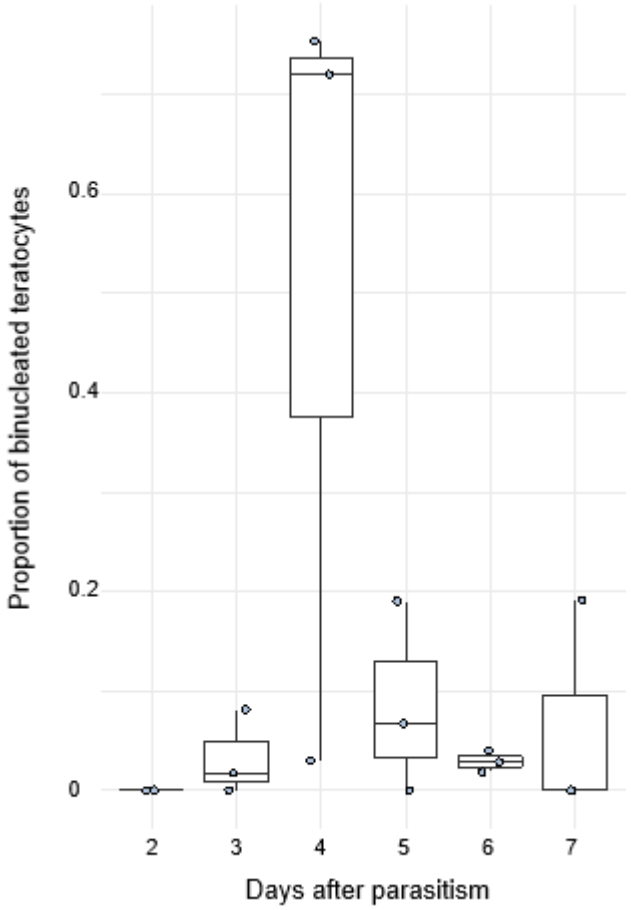


Figure 4. Average area of teratocytes of *Trichopria drosophilae* per pupae of *Drosophila melanogaster*.



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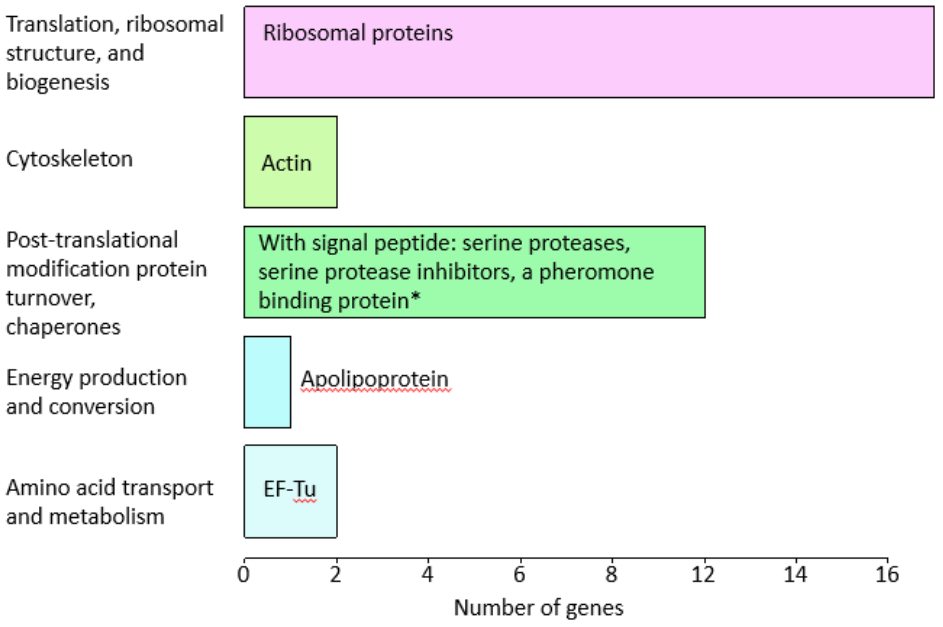
525 Figure 5. Proportion of binucleated teratocytes of *Trichopria drosophilae* in pupae of

526 *Drosophila melanogaster*



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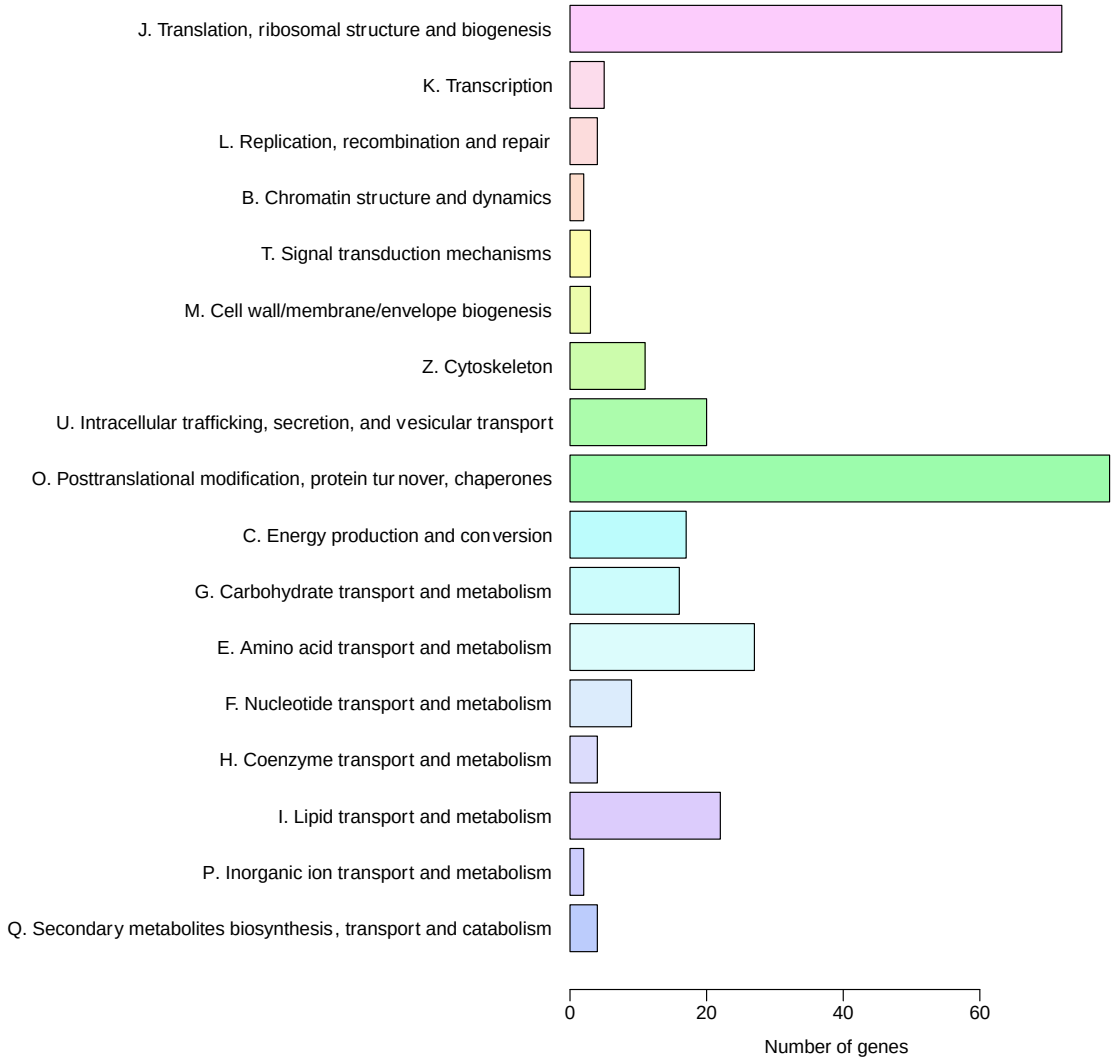
528 Figure 6. Number of genes of *Trichopria drosophilae* teratocytes with 1000-9999 TPM.



529

530 Figure 7. Number of genes of *Trichopria drosophilae* teratocytes with 100-999 TPM.

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Supplementary material

Table S1: Developmental of *Drosophila melanogaster* pupae injected with different amount of venom of *Trichopria drosophilae*. VR: Venom reservouir equivalent

	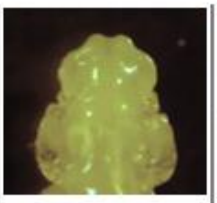	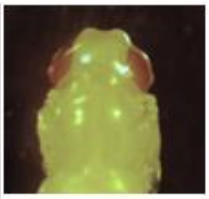		Emergence
0.2 VR/pupa	100%	50%	5%	0%
0.1 VR/pupa	100%	75%	5%	0%
0.05 VR/pupa	100%	100%	95%	0%
0.025 VR/pupa	100%	100%	95%	0%
PBS	100%	100%	100%	100%

Table S2. Proteins identified in the venom proteome of *Trichopria drosophilae*.

Id Trinity	Length	Hits	Putative protein
TRINITY_DN55_c0_g1_i1.p1	871	805	Aminopeptidase N-like
TRINITY_DN3856_c1_g1_i10.p1	641	460	Ioricrin-like
TRINITY_DN432_c0_g1_i7.p1	1152	445	hypothetical protein [Biostraticola sp. BGMRC 2031]
TRINITY_DN55_c0_g2_i2.p1	882	290	Aminopeptidase N-like
TRINITY_DN2295_c0_g1_i1.p1	482	270	Conserved Plasmodium protein, unknown function
TRINITY_DN3823_c0_g1_i2.p1	1161	270	Hypothetical protein [Sodalis sp. 159R]
TRINITY_DN158_c0_g4_i2.p1	703	207	neprilysin-2-like isoform X3
TRINITY_DN158_c0_g2_i1.p1	743	206	neprilysin-2 isoform X1
TRINITY_DN432_c0_g1_i14.p1	1143	200	Hypothetical protein [Arsenophonus endosymbiont of Nilaparvata lugens]
TRINITY_DN5077_c0_g1_i1.p1	1054	199	Hypothetical protein [Sodalis sp.]
TRINITY_DN3114_c1_g1_i1.p1	571	199	uncharacterized protein LOC111508756 [Leptinotarsa decemlineata]
TRINITY_DN1977_c0_g1_i1.p1	3384	192	Apolipophorins

TRINITY_DN1384_c0_g1_i1.p1	261	183	LOW QUALITY PROTEIN: collagen alpha-1(XVIII) chain
TRINITY_DN2826_c0_g1_i3.p1	697	175	endoplasmic reticulum chaperone BiP isoform X2
TRINITY_DN996_c0_g2_i1.p1	656	158	pentapeptide repeat-containing protein [Arsenophonus endosymbiont of Nilaparvata lugens]
TRINITY_DN2837_c0_g1_i1.p1	577	147	uncharacterized protein LOC108906945 [Anoplophora glabripennis]
TRINITY_DN3004_c0_g1_i1.p1	515	147	endonuclease-like venom protein precursor [Nasonia vitripennis]
TRINITY_DN1732_c0_g2_i2.p1	2416	137	fatty acid synthase [Cephus cinctus]
TRINITY_DN3544_c0_g3_i2.p1	1019	133	hypothetical protein BG55_20140 [Erwinia mallotivora]
TRINITY_DN5385_c0_g1_i1.p1	718	131	neprilysin-2 isoform X1 [Athalia rosae]
TRINITY_DN2135_c0_g1_i1.p1	577	127	uncharacterized protein LOC100678309 isoform X3 [Nasonia vitripennis]
TRINITY_DN1637_c0_g1_i7.p1	528	117	endonuclease-like venom protein precursor [Nasonia vitripennis]
TRINITY_DN23022_c0_g1_i1.p1	1143	115	hypothetical protein [Sodalis praecaptivus]
TRINITY_DN207_c0_g1_i16.p1	1969	110	myosin heavy chain, muscle isoform X8 [Ceratosolen solmsi marchali]
TRINITY_DN1932_c0_g1_i31.p1	2391	94	fatty acid synthase [Nasonia vitripennis]
TRINITY_DN158_c1_g1_i4.p1	712	86	membrane metallo-endopeptidase-like 1 isoform X2 [Ceratosolen solmsi marchali]
TRINITY_DN2336_c0_g1_i2.p1	633	86	beta-galactosidase-like isoform X1 [Nasonia vitripennis]
TRINITY_DN2914_c0_g1_i1.p1	574	86	toxin PirB [Pectobacterium]
TRINITY_DN4927_c0_g1_i1.p1	795	85	endoplasmin [Nasonia vitripennis]
TRINITY_DN140_c0_g1_i1.p1	708	84	transferrin isoform X1 [Orussus abietinus]
TRINITY_DN2473_c0_g1_i3.p1	436	80	venom metalloproteinase 3-like [Wasmannia auropunctata]

TRINITY_DN1410_c0_g1_i1.p1	487	79	AAA family ATPase [Clostridium saccharobutylicum]
TRINITY_DN11833_c0_g1_i1.p1	711	76	arylphorin subunit alpha-like [Nasonia vitripennis]
TRINITY_DN996_c0_g1_i1.p1	543	72	hypothetical protein [Arsenophonus endosymbiont of Nilaparvata lugens]
TRINITY_DN996_c0_g3_i2.p1	545	70	hypothetical protein [Rickettsia hoogstraalii]
TRINITY_DN5624_c0_g1_i1.p1	835	70	hypothetical protein [Pectobacterium]
TRINITY_DN14507_c0_g1_i1.p1	1168	67	chitotriosidase-1 isoform X2 [Nasonia vitripennis]
TRINITY_DN1801_c0_g1_i1.p1	581	65	uncharacterized protein LOC111517505 [Leptinotarsa decemlineata]
TRINITY_DN1637_c0_g1_i9.p1	529	63	endonuclease-like venom protein precursor [Nasonia vitripennis]
TRINITY_DN1315_c0_g1_i2.p1	908	61	hypothetical protein TSAR_000707 [Trichomalopsis sarcophagae]
TRINITY_DN1304_c0_g1_i2.p1	198	55	metalloproteinase inhibitor 2 isoform X2 [Dufourea novaeangliae]
TRINITY_DN1946_c0_g1_i2.p1	453	53	A disintegrin and metalloproteinase with thrombospondin motifs 12 [Diachasma alloeum]
TRINITY_DN5325_c0_g1_i2.p1	382	51	hypothetical protein DBV15_00338 [Temnothorax longispinosus]
TRINITY_DN34_c0_g1_i1.p1	230	51	No significant similarity found
TRINITY_DN360_c0_g1_i1.p1	396	49	serpin-1K [Pteromalus puparum]
TRINITY_DN893_c0_g1_i8.p1	377	48	beta-actin [Cotesia chilonis]
TRINITY_DN3098_c0_g1_i26.p1	448	46	A disintegrin and metalloproteinase with thrombospondin motifs 12 [Diachasma alloeum]
TRINITY_DN4177_c0_g1_i1.p1	439	46	A disintegrin and metalloproteinase with thrombospondin motifs 5-like [Microplitis demolitor]
TRINITY_DN2909_c0_g1_i1.p1	2401	43	fatty acid synthase [Nasonia vitripennis]

TRINITY_DN5599_c0_g1_i1.p1	174	42	hypothetical protein CPT96_03200 [Candidatus Gastranaerophilales bacterium HUM_10]
TRINITY_DN4277_c0_g1_i2.p1	264	41	No significant similarity found
TRINITY_DN2324_c0_g1_i2.p1	463	41	hypothetical protein TSAR_008633 [Trichomalopsis sarcophagae]
TRINITY_DN2246_c0_g1_i1.p1	894	41	tudor staphylococcus/micrococcal nuclease [Nasonia vitripennis]
TRINITY_DN4661_c0_g1_i1.p1	492	40	protein disulfide-isomerase A3 [Cephus cinctus]
TRINITY_DN1373_c0_g1_i1.p1	112	40	SDR family NAD(P)-dependent oxidoreductase [Streptomyces sp. SID7982]
TRINITY_DN1289_c0_g1_i6.p1	878	39	paramyosin, long form [Harpegnathos saltator]
TRINITY_DN5591_c0_g1_i2.p1	534	39	matrix metalloproteinase-14 isoform X5 [Trachymyrmex septentrionalis]
TRINITY_DN1637_c0_g1_i2.p1	529	37	endonuclease-like venom protein precursor [Nasonia vitripennis]
TRINITY_DN4277_c0_g1_i5.p1	120	37	Chain A, cellulase [Hungateiclostridium cellulolyticum]
TRINITY_DN1254_c0_g1_i1.p1	887	37	putative translation elongation factor 2 [Cotesia chilonis]
TRINITY_DN163_c0_g1_i1.p1	617	37	V-type proton ATPase catalytic subunit A [Neodiprion lecontei]
TRINITY_DN23_c0_g1_i6.p1	374	36	mucin-2-like isoform X2 [Cyphomyrmex costatus]
TRINITY_DN3910_c0_g1_i1.p1	213	36	uncharacterized protein LOC106685315 [Halyomorpha halys]
TRINITY_DN1743_c0_g1_i3.p1	357	35	hypothetical protein AUJ10_00985 [Candidatus Pacearchaeota archaeon CG1_02_31_27]
TRINITY_DN6233_c0_g1_i1.p1	214	34	metalloproteinase inhibitor 1 isoform X5 [Acromyrmex echinatior]

TRINITY_DN31332_c0_g1_i1.p1	1531	34	UDP-glucose:glycoprotein glucosyltransferase isoform X1 [Cephus cinctus]
TRINITY_DN5978_c0_g1_i3.p1	269	33	GHKL domain-containing protein [Shewanella psychrophila]
TRINITY_DN3984_c0_g1_i3.p1	587	32	hypothetical protein [Borrelia hermsii]
TRINITY_DN9526_c0_g1_i1.p1	418	32	venom acid phosphatase Acph-1 [Harpegnathos saltator]
TRINITY_DN914_c0_g1_i1.p1	457	32	enolase isoform X2 [Microplitis demolitor]
TRINITY_DN1475_c0_g1_i2.p1	154	31	No significant similarity found
TRINITY_DN3742_c0_g1_i1.p1	316	31	pancreatic lipase-related protein 2 isoform X3 [Monomorium pharaonis]
TRINITY_DN19972_c0_g1_i1.p1	157	30	general odorant-binding protein 56d [Nasonia vitripennis]
TRINITY_DN557_c3_g1_i1.p1	334	30	glyceraldehyde-3-phosphate dehydrogenase 2 [Trichogramma pretiosum]
TRINITY_DN4836_c1_g2_i3.p1	722	28	heat shock protein 83 [Orussus abietinus]
TRINITY_DN1694_c0_g1_i1.p1	1197	28	pyruvate carboxylase, mitochondrial isoform X1 [Apis cerana]
TRINITY_DN4540_c0_g1_i2.p1	428	26	A disintegrin and metalloproteinase with thrombospondin motifs 5-like [Microplitis demolitor]
TRINITY_DN2834_c0_g2_i1.p1	424	25	venom metalloproteinase 3-like [Trichogramma pretiosum]
TRINITY_DN79_c0_g1_i3.p1	366	25	fructose-bisphosphate aldolase-like isoform X2 [Ceratina calcarata]
TRINITY_DN7864_c1_g1_i1.p1	661	24	heat shock 70 kDa protein cognate 4 [Camponotus floridanus]
TRINITY_DN3666_c0_g1_i1.p1	726	24	hypothetical protein TSAR_002130 [Trichomalopsis sarcophagae]
TRINITY_DN5020_c0_g1_i1.p1	512	24	CLUMA_CG008519, isoform C [Clunio marinus]
TRINITY_DN432_c1_g2_i1.p1	244	23	No significant similarity found

TRINITY_DN4580_c0_g1_i1.p1	243	23	No significant similarity found
TRINITY_DN432_c0_g2_i1.p1	307	23	tubulin beta-1 chain [Cephus cinctus]
TRINITY_DN1590_c0_g1_i1.p1	789	23	aconitate hydratase, mitochondrial [Orussus abietinus]
TRINITY_DN7804_c0_g1_i3.p1	713	23	membrane metallo-endopeptidase-like 1 [Fopius arisanus]
TRINITY_DN4891_c0_g1_i2.p1	419	22	snake venom metalloproteinase BjussuMP-2-like [Temnothorax curvispinosus]
TRINITY_DN60_c0_g2_i1.p1	451	22	tubulin alpha-1 chain [Neodiprion lecontei]
TRINITY_DN711_c0_g1_i1.p1	1110	22	ubiquitin-like modifier-activating enzyme 1 [Megachile rotundata]
TRINITY_DN752_c0_g1_i2.p1	147	22	No significant similarity found
TRINITY_DN3115_c0_g1_i2.p1	423	21	A disintegrin and metalloproteinase with thrombospondin motifs 5-like [Microplitis demolitor]
TRINITY_DN249_c0_g1_i1.p1	623	21	transketolase isoform X1 [Nasonia vitripennis]
TRINITY_DN3925_c0_g1_i2.p1	330	21	uncharacterized peptidase C1-like protein F26E4.3 [Rhopalosiphum maidis]
TRINITY_DN700_c0_g2_i2.p1	1727	20	filamin-A isoform X1 [Nasonia vitripennis]
TRINITY_DN318_c0_g1_i2.p1	892	20	alpha-actinin, sarcomeric isoform X2 [Dufourea novaeangliae]
TRINITY_DN195_c0_g1_i14.p1	249	20	14-3-3 protein zeta isoform X1 [Nasonia vitripennis]
TRINITY_DN4538_c0_g1_i2.p1	333	20	uncharacterized protein LOC103571167 [Microplitis demolitor]
TRINITY_DN4345_c0_g1_i4.p1	398	20	uncharacterized protein LOC106659934 [Trichogramma pretiosum]
TRINITY_DN35559_c0_g1_i1.p1	247	20	venom allergen 3-like [Trichogramma pretiosum]
TRINITY_DN27683_c0_g1_i1.p1	840	19	Transitional endoplasmic reticulum ATPase TER94 [Cyphomyrmex costatus]
TRINITY_DN7649_c0_g1_i1.p1	617	19	arylsulfatase I-like [Copidosoma floridanum]

TRINITY_DN2108_c0_g1_i1.p1	844	19	glycogen phosphorylase [Nasonia vitripennis]
TRINITY_DN1160_c0_g1_i1.p1	507	19	matrix metalloproteinase-14 isoform X1 [Cephus cinctus]
TRINITY_DN23141_c0_g1_i1.p1	519	19	ATP synthase subunit beta, mitochondrial [Trichogramma pretiosum]
TRINITY_DN6227_c2_g1_i1.p1	444	19	calreticulin [Cephus cinctus]
TRINITY_DN10923_c0_g1_i1.p1	152	19	armadillo repeat-containing protein 2 isoform X2 [Mus musculus]
TRINITY_DN777_c0_g1_i1.p1	173	18	QWxxN domain [Enterococcus durans]
TRINITY_DN5587_c0_g1_i1.p1	290	18	stromelysin-1-like [Pseudonaja textilis]
TRINITY_DN11392_c0_g1_i2.p1	236	18	venom allergen 3-like [Trichogramma pretiosum]
TRINITY_DN368_c0_g2_i1.p1	691	17	diphenol oxidase 3 [Nasonia vitripennis]
TRINITY_DN2667_c0_g1_i2.p1	570	17	moesin/ezrin/radixin homolog 1 isoform X2 [Nasonia vitripennis]
TRINITY_DN3397_c0_g1_i8.p1	551	17	esterase B1 isoform X1 [Cephus cinctus]
TRINITY_DN39753_c0_g1_i1.p1	403	17	hypothetical protein TSAR_004134 [Trichomalopsis sarcophagae]
TRINITY_DN10847_c0_g1_i3.p1	534	17	matrix metalloproteinase-14 isoform X4 [Trachymyrmex cornetzi]
TRINITY_DN636_c0_g1_i2.p1	694	17	hypothetical protein TSAR_002489 [Trichomalopsis sarcophagae]
TRINITY_DN645_c0_g1_i7.p1	2379	16	spectrin beta chain isoform X2 [Orussus abietinus]
TRINITY_DN39681_c0_g1_i1.p1	828	16	hypothetical protein TSAR_004117 [Trichomalopsis sarcophagae]
TRINITY_DN288_c0_g1_i1.p1	613	16	dihydropyrimidinase isoform X1 [Orussus abietinus]
TRINITY_DN192_c0_g1_i2.p1	324	16	annexin B9 isoform X3 [Nasonia vitripennis]
TRINITY_DN2899_c0_g1_i1.p1	673	16	endothelin-converting enzyme 1-like [Ceratosolen solmsi marchali]

TRINITY_DN3983_c0_g1_i1.p1	191	15	pentapeptide repeat-containing protein [Arsenophonus endosymbiont of Nilaparvata lugens]
TRINITY_DN35392_c0_g1_i1.p1	549	15	ATP synthase subunit alpha, mitochondrial [Camponotus floridanus]
TRINITY_DN1598_c0_g1_i1.p1	687	15	heat shock 70 kDa protein cognate 5 [Osmia bicornis bicornis]
TRINITY_DN2332_c0_g1_i5.p1	496	15	protein disulfide-isomerase [Wasmannia auropunctata]
TRINITY_DN3869_c0_g1_i8.p1	566	15	esterase E4 [Nasonia vitripennis]
TRINITY_DN5910_c0_g1_i1.p1	301	15	ADP,ATP carrier protein 2 [Copidosoma floridanum]
TRINITY_DN39773_c0_g1_i1.p1	256	15	3-hydroxyacyl-CoA dehydrogenase type-2 [Nasonia vitripennis]
TRINITY_DN5167_c0_g1_i4.p1	282	15	vitronectin-like [Echeneis naucrates]
TRINITY_DN3024_c0_g1_i1.p1	111	14	No significant similarity found
TRINITY_DN2077_c5_g1_i1.p1	578	14	hypothetical protein TSAR_004660 [Trichomalopsis sarcophagae]
TRINITY_DN2888_c0_g1_i1.p1	484	14	hypothetical protein TSAR_005593 [Trichomalopsis sarcophagae]
TRINITY_DN40896_c0_g1_i1.p2	161	14	Myh9 protein [Mus musculus]
TRINITY_DN1307_c0_g1_i2.p1	1003	13	calcium-transporting ATPase sarcoplasmic/endoplasmic reticulum type isoform X3 [Nylanderia fulva]
TRINITY_DN15116_c0_g1_i1.p1	399	13	hypothetical protein [Saprospirales bacterium]
TRINITY_DN3982_c0_g1_i1.p1	203	13	glutathione S-transferase S3 [Nasonia vitripennis]
TRINITY_DN4843_c0_g1_i1.p1	220	13	cathepsin L1-like isoform X3 [Python bivittatus]
TRINITY_DN368_c0_g1_i1.p1	698	13	phenoloxidase 2-like [Eufriesea mexicana]
TRINITY_DN3983_c0_g1_i2.p1	74	13	DUF1217 domain-containing protein [Rhizobium leguminosarum]

TRINITY_DN9642_c0_g1_i1.p1	410	13	hypothetical protein DD595_26355 [Enterobacter cloacae complex sp. 4DZ3-17B2]
TRINITY_DN158_c1_g1_i2.p1	724	12	neprilysin-2-like isoform X3 [Nasonia vitripennis]
TRINITY_DN1261_c0_g1_i1.p1	1679	12	clathrin heavy chain [Camponotus floridanus]
TRINITY_DN1066_c0_g1_i1.p1	1459	12	CD109 antigen isoform X3 [Nasonia vitripennis]
TRINITY_DN1959_c0_g1_i1.p1	1032	12	hypothetical protein TSAR_002631 [Trichomalopsis sarcophagae]
TRINITY_DN19025_c0_g1_i1.p1	626	12	polyadenylate-binding protein 1 [Nomia melanderi]
TRINITY_DN14283_c0_g1_i1.p1	448	12	tubulin beta-1 chain [Cephus cinctus]
TRINITY_DN2062_c0_g1_i1.p1	632	12	very long-chain specific acyl-CoA dehydrogenase, mitochondrial isoform X1 [Cephus cinctus]
TRINITY_DN28093_c0_g1_i1.p1	139	12	uncharacterized protein LOC105593202 [Cercocebus atys]
TRINITY_DN5444_c0_g1_i1.p1	208	12	putative Per a allergen [Periplaneta americana]
TRINITY_DN3920_c0_g1_i1.p1	262	12	40S ribosomal protein S3 [Nasonia vitripennis]
TRINITY_DN6596_c0_g1_i1.p1	324	12	
TRINITY_DN32335_c0_g1_i1.p1	252	12	venom allergen 3-like [Trichogramma pretiosum]
TRINITY_DN4733_c0_g1_i5.p1	416	12	
TRINITY_DN9922_c0_g3_i2.p1	396	11	uncharacterized protein LOC100679457 [Nasonia vitripennis]
TRINITY_DN23050_c0_g1_i1.p1	579	11	beta-hexosaminidase subunit beta [Nasonia vitripennis]

TRINITY_DN1822_c0_g1_i1.p1	601	11	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 1 [Cephus cinctus]
TRINITY_DN713_c1_g1_i4.p1	1194	11	protein transport protein Sec24C isoform X1 [Cephus cinctus]
TRINITY_DN2966_c0_g1_i1.p1	429	11	
TRINITY_DN3548_c0_g1_i1.p1	766	11	
TRINITY_DN1817_c0_g1_i26.p1	776	11	
TRINITY_DN18958_c0_g1_i1.p1	197	11	
TRINITY_DN31613_c0_g1_i1.p1	218	11	
TRINITY_DN3348_c0_g1_i1.p1	930	10	alpha glucosidase II alpha subunit-like precursor [Nasonia vitripennis]
TRINITY_DN2077_c1_g1_i7.p1	818	10	alpha,alpha-trehalose-phosphate synthase [UDP-forming] isoform X3 [Cephus cinctus]
TRINITY_DN1209_c1_g1_i4.p2	1103	10	spectrin alpha chain isoform X2 [Bombus terrestris]
TRINITY_DN27681_c0_g1_i1.p1	392	10	ovalbumin-related protein X isoform X15 [Nasonia vitripennis]
TRINITY_DN7146_c1_g1_i4.p1	424	10	venom metalloproteinase 3-like isoform X3 [Nasonia vitripennis]
TRINITY_DN1055_c0_g1_i2.p1	802	10	prolyl endopeptidase [Osmia bicornis bicornis]
TRINITY_DN1616_c0_g1_i1.p2	156	10	No significant similarity found
TRINITY_DN1903_c0_g1_i1.p1	483	10	synaptic vesicle membrane protein VAT-1 homolog-like [Polistes canadensis]
TRINITY_DN3264_c0_g1_i1.p1	562	10	vanin-like protein 1 isoform X1 [Nasonia vitripennis]
TRINITY_DN2392_c0_g1_i1.p1	459	10	
TRINITY_DN1209_c1_g1_i4.p1	1348	9	spectrin alpha chain [Orussus abietinus]
TRINITY_DN18830_c0_g1_i1.p1	443	9	hypothetical protein TSAR_006419 [Trichomalopsis sarcophagae]
TRINITY_DN19267_c0_g1_i1.p1	367	9	aldo-keto reductase family 1 member A1-like [Nasonia vitripennis]

TRINITY_DN4713_c0_g1_i1.p1	537	9	
TRINITY_DN3355_c1_g1_i1.p1	248	9	
TRINITY_DN5500_c0_g1_i2.p1	681	9	
TRINITY_DN802_c1_g3_i2.p1	688	9	
TRINITY_DN989_c0_g2_i1.p1	626	9	
TRINITY_DN31592_c0_g1_i1.p1	410	9	
TRINITY_DN2310_c0_g1_i1.p1	505	9	
TRINITY_DN27911_c0_g1_i1.p1	490	9	
TRINITY_DN2602_c0_g1_i10.p1	250	9	
TRINITY_DN1498_c0_g1_i1.p1	418	9	
TRINITY_DN51_c0_g1_i1.p1	875	9	
TRINITY_DN1118_c0_g1_i12.p2	374	9	
TRINITY_DN31318_c0_g1_i1.p2	389	9	
TRINITY_DN2447_c0_g1_i1.p1	291	9	
TRINITY_DN1229_c0_g1_i3.p1	2068	8	putative glutamate synthase [NADPH] isoform X1 [Cephus cinctus]
TRINITY_DN5326_c0_g1_i2.p1	599	8	lamin Dm0 isoform X2 [Orussus abietinus]
TRINITY_DN1644_c0_g1_i20.p1	614	8	
TRINITY_DN4148_c0_g1_i2.p1	612	8	
TRINITY_DN70_c0_g1_i4.p2	277	8	
TRINITY_DN144_c0_g1_i1.p1	449	8	
TRINITY_DN4394_c0_g1_i1.p1	607	8	
TRINITY_DN1127_c0_g2_i2.p2	221	8	
TRINITY_DN39888_c0_g1_i1.p1	149	8	
TRINITY_DN3888_c0_g2_i1.p1	209	8	
TRINITY_DN4318_c0_g3_i1.p1	582	8	
TRINITY_DN329_c0_g1_i14.p1	325	7	annexin B9 [Orussus abietinus]
TRINITY_DN3753_c0_g1_i1.p1	226	7	conserved Plasmodium protein, unknown function [Plasmodium sp. gorilla clade G3]
TRINITY_DN2867_c0_g1_i2.p1	274	7	
TRINITY_DN3182_c0_g1_i1.p1	1216	7	
TRINITY_DN3199_c0_g3_i1.p1	281	7	
TRINITY_DN210_c0_g1_i12.p1	285	7	

TRINITY_DN5987_c0_g2_i1.p1	333	7
TRINITY_DN1743_c0_g1_i7.p1	421	7
TRINITY_DN4620_c0_g1_i2.p1	698	7
TRINITY_DN39717_c0_g1_i1.p1	464	7
TRINITY_DN4327_c0_g1_i3.p1	518	7
TRINITY_DN8246_c0_g1_i5.p1	237	7
TRINITY_DN3477_c0_g1_i1.p1	339	7
TRINITY_DN592_c0_g1_i1.p1	1024	7
TRINITY_DN19344_c0_g1_i1.p1	185	7
TRINITY_DN27961_c0_g1_i1.p2	272	7
TRINITY_DN23087_c0_g1_i1.p1	165	7
TRINITY_DN23589_c0_g1_i1.p1	263	7
TRINITY_DN2933_c0_g1_i1.p1	515	6
TRINITY_DN3089_c0_g1_i3.p1	1241	6
TRINITY_DN2856_c0_g1_i9.p1	1565	6
TRINITY_DN1690_c0_g1_i1.p1	196	6
TRINITY_DN1565_c0_g1_i1.p1	285	6
TRINITY_DN1866_c0_g1_i1.p1	333	6
TRINITY_DN2980_c0_g1_i2.p1	321	6
TRINITY_DN3343_c0_g1_i1.p1	1161	6
TRINITY_DN16180_c0_g1_i1.p1	390	6
TRINITY_DN2112_c0_g1_i1.p1	824	6
TRINITY_DN299_c1_g1_i15.p1	449	6
TRINITY_DN490_c0_g1_i1.p1	341	6
TRINITY_DN2098_c0_g1_i1.p1	567	6
TRINITY_DN3800_c0_g1_i5.p1	258	6
TRINITY_DN6546_c0_g1_i1.p1	159	6
TRINITY_DN5167_c0_g1_i6.p1	279	6
TRINITY_DN11034_c0_g1_i1.p1	335	6
TRINITY_DN1465_c0_g1_i1.p1	433	6
TRINITY_DN19233_c0_g1_i1.p2	315	6
TRINITY_DN2866_c0_g1_i1.p1	427	6

TRINITY_DN32354_c0_g1_i1.p1	298	6	
TRINITY_DN4418_c0_g1_i5.p1	686	6	
TRINITY_DN20180_c0_g1_i1.p1	164	6	
TRINITY_DN2275_c0_g1_i1.p1	399	6	
TRINITY_DN39581_c0_g1_i1.p2	156	6	
TRINITY_DN679_c0_g1_i11.p3	262	6	
TRINITY_DN1109_c0_g1_i2.p1	1170	5	vigilin [Agrilus planipennis]
TRINITY_DN8406_c0_g1_i1.p1	5839	5	titin-like isoform X4 [Megachile rotundata]
TRINITY_DN864_c0_g2_i2.p1	1963	5	myosin heavy chain, non-muscle isoform X5 [Copidosoma floridanum]
TRINITY_DN35415_c0_g1_i1.p1	1018	5	
TRINITY_DN12_c7_g1_i1.p2	265	5	
TRINITY_DN1071_c0_g1_i3.p1	1107	5	
TRINITY_DN39835_c0_g1_i1.p1	548	5	
TRINITY_DN4928_c0_g1_i1.p1	539	5	
TRINITY_DN909_c0_g1_i1.p1	948	5	
TRINITY_DN2456_c0_g1_i1.p1	414	5	
TRINITY_DN35819_c0_g1_i1.p1	315	5	
TRINITY_DN40106_c0_g1_i1.p1	593	5	
TRINITY_DN92_c0_g1_i3.p1	198	5	
TRINITY_DN2833_c0_g1_i4.p1	672	5	
TRINITY_DN837_c0_g1_i1.p1	148	5	
TRINITY_DN4809_c0_g1_i1.p1	1234	5	
TRINITY_DN211_c0_g1_i6.p1	211	5	
TRINITY_DN1192_c0_g1_i5.p1	780	5	
TRINITY_DN23254_c0_g1_i1.p1	536	5	
TRINITY_DN27388_c0_g1_i1.p1	439	5	
TRINITY_DN3943_c0_g1_i1.p1	436	5	
TRINITY_DN39615_c0_g1_i1.p1	306	5	
TRINITY_DN5962_c0_g1_i1.p1	731	5	
TRINITY_DN5_c0_g1_i1.p1	320	5	
TRINITY_DN6636_c0_g2_i3.p1	111	5	

TRINITY_DN5523_c0_g1_i44.p2	354	5	
TRINITY_DN1286_c1_g1_i1.p1	135	5	
TRINITY_DN13_c0_g1_i1.p1	394	5	
TRINITY_DN23366_c0_g1_i1.p1	215	5	
TRINITY_DN36165_c0_g1_i1.p1	304	5	
TRINITY_DN18959_c0_g1_i1.p1	385	5	
TRINITY_DN2469_c0_g1_i1.p2	182	5	
TRINITY_DN3280_c0_g1_i1.p1	289	5	
TRINITY_DN717_c0_g1_i1.p1	528	5	
TRINITY_DN3511_c0_g1_i1.p1	1788	4	laminin subunit beta-1 [Copidosoma floridanum]
TRINITY_DN689_c0_g1_i4.p1	194	4	
TRINITY_DN527_c0_g1_i3.p1	931	4	
TRINITY_DN939_c0_g1_i2.p1	1266	4	
TRINITY_DN2024_c0_g1_i1.p1	958	4	
TRINITY_DN39581_c0_g1_i1.p1	557	4	
TRINITY_DN28695_c0_g1_i1.p1	358	4	
TRINITY_DN4111_c0_g1_i1.p1	391	4	
TRINITY_DN1172_c0_g1_i4.p1	521	4	
TRINITY_DN1757_c0_g1_i1.p1	746	4	
TRINITY_DN2745_c0_g1_i1.p1	620	4	
TRINITY_DN1286_c0_g1_i3.p1	125	4	
TRINITY_DN3300_c0_g1_i2.p1	1325	4	
TRINITY_DN1909_c0_g1_i1.p1	318	4	
TRINITY_DN1922_c0_g1_i1.p1	1059	4	
TRINITY_DN19594_c0_g1_i1.p1	203	4	
TRINITY_DN907_c0_g1_i2.p2	248	4	
TRINITY_DN27694_c0_g1_i1.p1	145	4	
TRINITY_DN1016_c0_g1_i2.p2	132	4	
TRINITY_DN28041_c0_g1_i1.p1	295	4	
TRINITY_DN2970_c0_g1_i1.p1	1075	4	
TRINITY_DN3325_c0_g1_i2.p2	1019	4	

TRINITY_DN3641_c0_g1_i2.p1	1241	4
TRINITY_DN37228_c0_g1_i1.p1	191	4
TRINITY_DN679_c0_g1_i1.p1	313	4
TRINITY_DN576_c0_g1_i2.p1	183	4
TRINITY_DN4227_c0_g1_i1.p1	262	4
TRINITY_DN4252_c0_g1_i1.p1	109	4
TRINITY_DN67_c0_g1_i3.p1	548	4
TRINITY_DN39862_c0_g1_i1.p1	364	4
TRINITY_DN1342_c0_g1_i2.p1	127	4
TRINITY_DN1081_c0_g1_i3.p1	318	4
TRINITY_DN24556_c0_g1_i1.p1	296	4
TRINITY_DN37013_c0_g1_i1.p1	123	4
TRINITY_DN530_c0_g2_i1.p1	593	4
TRINITY_DN1579_c0_g1_i3.p1	320	4
TRINITY_DN19215_c0_g1_i1.p1	289	4
TRINITY_DN20744_c0_g1_i1.p1	178	4
TRINITY_DN23009_c0_g1_i1.p1	333	4
TRINITY_DN2314_c0_g1_i3.p2	183	4
TRINITY_DN2658_c0_g3_i1.p1	211	4
TRINITY_DN3960_c0_g1_i1.p1	425	4
TRINITY_DN1277_c0_g1_i3.p1	254	4
TRINITY_DN32465_c0_g1_i1.p1	265	4
TRINITY_DN40269_c0_g1_i1.p1	200	4
TRINITY_DN516_c0_g1_i10.p1	252	4
TRINITY_DN218_c0_g1_i1.p1	200	4
TRINITY_DN1487_c0_g2_i1.p1	376	4
TRINITY_DN2023_c0_g1_i3.p2	286	4
TRINITY_DN31339_c0_g1_i1.p1	250	4
TRINITY_DN566_c0_g1_i1.p1	194	4
TRINITY_DN8986_c0_g1_i2.p1	320	4
TRINITY_DN4109_c0_g1_i1.p1	646	4
TRINITY_DN5417_c0_g1_i2.p1	215	4

TRINITY_DN20084_c0_g1_i1.p1	206	4	
TRINITY_DN4475_c1_g1_i1.p1	211	3	endonuclease-like venom protein precursor [Nasonia vitripennis]
TRINITY_DN3889_c0_g1_i1.p1	640	3	heat shock 70 kDa protein cognate 4 isoform X1 [Wasmannia auropunctata]
TRINITY_DN8928_c0_g1_i1.p1	2713	3	laminin subunit alpha [Nasonia vitripennis]
TRINITY_DN1309_c0_g2_i10.p1	641	3	
TRINITY_DN1703_c0_g1_i1.p1	938	3	
TRINITY_DN2207_c0_g1_i1.p1	396	3	
TRINITY_DN1464_c0_g1_i1.p1	1020	3	
TRINITY_DN3336_c0_g1_i1.p1	415	3	
TRINITY_DN82_c0_g1_i3.p1	496	3	
TRINITY_DN4852_c0_g1_i1.p1	217	3	
TRINITY_DN69_c0_g2_i4.p1	425	3	
TRINITY_DN605_c0_g1_i1.p1	358	3	
TRINITY_DN921_c0_g1_i22.p1	621	3	
TRINITY_DN18896_c0_g1_i1.p1	472	3	
TRINITY_DN18997_c0_g1_i1.p1	494	3	
TRINITY_DN8168_c0_g1_i2.p1	477	3	
TRINITY_DN6893_c1_g1_i1.p1	768	3	
TRINITY_DN469_c0_g1_i4.p1	245	3	
TRINITY_DN1719_c0_g1_i1.p1	217	3	
TRINITY_DN1209_c2_g1_i3.p1	765	3	
TRINITY_DN1176_c0_g1_i3.p2	495	3	
TRINITY_DN1370_c0_g1_i2.p1	688	3	
TRINITY_DN3689_c0_g1_i1.p1	359	3	
TRINITY_DN848_c1_g1_i7.p1	286	3	
TRINITY_DN22985_c0_g1_i1.p1	335	3	
TRINITY_DN27415_c0_g1_i1.p2	263	3	
TRINITY_DN84_c0_g1_i11.p1	381	3	
TRINITY_DN881_c0_g1_i2.p1	558	3	
TRINITY_DN1627_c0_g1_i1.p1	202	3	
TRINITY_DN32064_c0_g1_i1.p1	189	3	

TRINITY_DN12_c5_g1_i1.p1	1202	3
TRINITY_DN1896_c0_g1_i4.p2	421	3
TRINITY_DN4980_c0_g1_i1.p1	278	3
TRINITY_DN910_c1_g1_i1.p1	503	3
TRINITY_DN1913_c1_g2_i3.p1	664	3
TRINITY_DN1728_c0_g1_i13.p1	233	3
TRINITY_DN2287_c0_g1_i1.p1	152	3
TRINITY_DN8241_c0_g1_i1.p1	592	3
TRINITY_DN23766_c0_g1_i1.p1	189	3
TRINITY_DN16_c6_g1_i4.p1	516	3
TRINITY_DN1817_c0_g1_i26.p3	254	3
TRINITY_DN4959_c0_g1_i2.p2	183	3
TRINITY_DN195_c1_g1_i2.p1	1683	3
TRINITY_DN39575_c0_g1_i1.p1	918	3
TRINITY_DN15238_c0_g1_i2.p1	522	3
TRINITY_DN35551_c0_g1_i1.p1	122	3
TRINITY_DN1169_c0_g1_i2.p1	416	3
TRINITY_DN2279_c0_g1_i16.p1	679	3
TRINITY_DN3811_c0_g1_i1.p1	383	3
TRINITY_DN504_c0_g1_i3.p1	443	3
TRINITY_DN5071_c0_g1_i1.p1	390	3
TRINITY_DN639_c0_g1_i1.p1	308	3
TRINITY_DN700_c0_g2_i13.p1	636	3
TRINITY_DN1451_c0_g1_i15.p1	1837	3
TRINITY_DN11119_c0_g1_i1.p1	383	3
TRINITY_DN4657_c0_g1_i1.p1	497	3
TRINITY_DN6503_c0_g1_i1.p1	106	3
TRINITY_DN22992_c0_g1_i1.p1	208	3
TRINITY_DN4270_c0_g1_i2.p1	281	3
TRINITY_DN3322_c0_g1_i1.p1	246	3
TRINITY_DN40120_c0_g1_i1.p1	133	3
TRINITY_DN3301_c0_g1_i1.p1	1097	3

TRINITY_DN5881_c0_g1_i14.p1	364	3
TRINITY_DN247_c0_g1_i2.p2	723	3
TRINITY_DN223_c0_g1_i9.p9	115	3
TRINITY_DN2137_c0_g1_i1.p1	170	3
TRINITY_DN4047_c0_g1_i1.p1	335	3
TRINITY_DN10343_c0_g1_i8.p1	263	3
TRINITY_DN1828_c0_g1_i1.p1	284	3
TRINITY_DN36048_c0_g1_i1.p1	319	3
TRINITY_DN39685_c0_g1_i1.p1	477	3
TRINITY_DN1708_c0_g1_i1.p1	299	3
TRINITY_DN23443_c0_g1_i1.p1	376	3
TRINITY_DN2878_c0_g1_i2.p1	710	3
TRINITY_DN299_c3_g1_i1.p1	227	3
TRINITY_DN40843_c0_g1_i1.p1	181	3
TRINITY_DN381_c0_g1_i2.p1	458	3
TRINITY_DN23142_c0_g1_i1.p1	580	3
TRINITY_DN5090_c0_g1_i1.p1	158	3
TRINITY_DN20526_c0_g1_i1.p1	114	3
TRINITY_DN756_c0_g1_i2.p1	564	3
TRINITY_DN7261_c0_g1_i1.p1	159	3
TRINITY_DN1792_c0_g1_i1.p1	243	3
TRINITY_DN2003_c0_g1_i1.p1	193	3
TRINITY_DN1310_c0_g1_i1.p2	258	3
TRINITY_DN40034_c0_g1_i1.p1	278	3
TRINITY_DN2099_c0_g1_i7.p1	481	3
TRINITY_DN5455_c0_g1_i1.p1	175	3
TRINITY_DN3968_c0_g1_i2.p2	160	3
TRINITY_DN2307_c0_g1_i1.p1	106	3
TRINITY_DN3661_c0_g1_i2.p1	186	3
TRINITY_DN2273_c0_g1_i1.p1	173	3
TRINITY_DN3003_c0_g1_i1.p1	1627	2
TRINITY_DN647_c0_g1_i14.p1	644	2

TRINITY_DN1192_c0_g1_i3.p1	505	2
TRINITY_DN312_c0_g2_i2.p1	2347	2
TRINITY_DN2754_c0_g1_i1.p1	480	2
TRINITY_DN103_c0_g1_i6.p1	1179	2
TRINITY_DN992_c0_g1_i13.p1	675	2
TRINITY_DN91_c0_g1_i1.p1	4288	2
TRINITY_DN809_c0_g2_i2.p1	2654	2
TRINITY_DN1127_c0_g1_i2.p1	351	2
TRINITY_DN1827_c0_g1_i1.p1	469	2
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TRINITY_DN3651_c0_g2_i2.p1	2241	2
TRINITY_DN630_c0_g1_i3.p1	562	2
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TRINITY_DN4218_c0_g1_i2.p1	675	2
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TRINITY_DN4676_c0_g1_i1.p1	413	2
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TRINITY_DN2991_c0_g1_i1.p1	581	2
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TRINITY_DN4446_c0_g1_i1.p1	306	2	
TRINITY_DN19338_c0_g1_i1.p1	160	2	
TRINITY_DN1302_c0_g1_i1.p1	334	2	
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TRINITY_DN1838_c0_g1_i2.p1	284	2	
TRINITY_DN1042_c0_g2_i2.p1	2391	1	fatty acid synthase [Nasonia vitripennis]
TRINITY_DN8159_c0_g1_i2.p1	247	1	
TRINITY_DN3764_c0_g1_i1.p1	935	1	
TRINITY_DN4318_c0_g1_i3.p1	581	1	
TRINITY_DN4318_c0_g2_i1.p1	579	1	
TRINITY_DN774_c0_g1_i1.p2	382	1	
TRINITY_DN839_c0_g1_i19.p1	922	1	
TRINITY_DN2187_c0_g1_i1.p1	201	1	
TRINITY_DN2881_c0_g1_i4.p1	526	1	major heat shock 70 kDa protein Ab-like [Microplitis demolitor]

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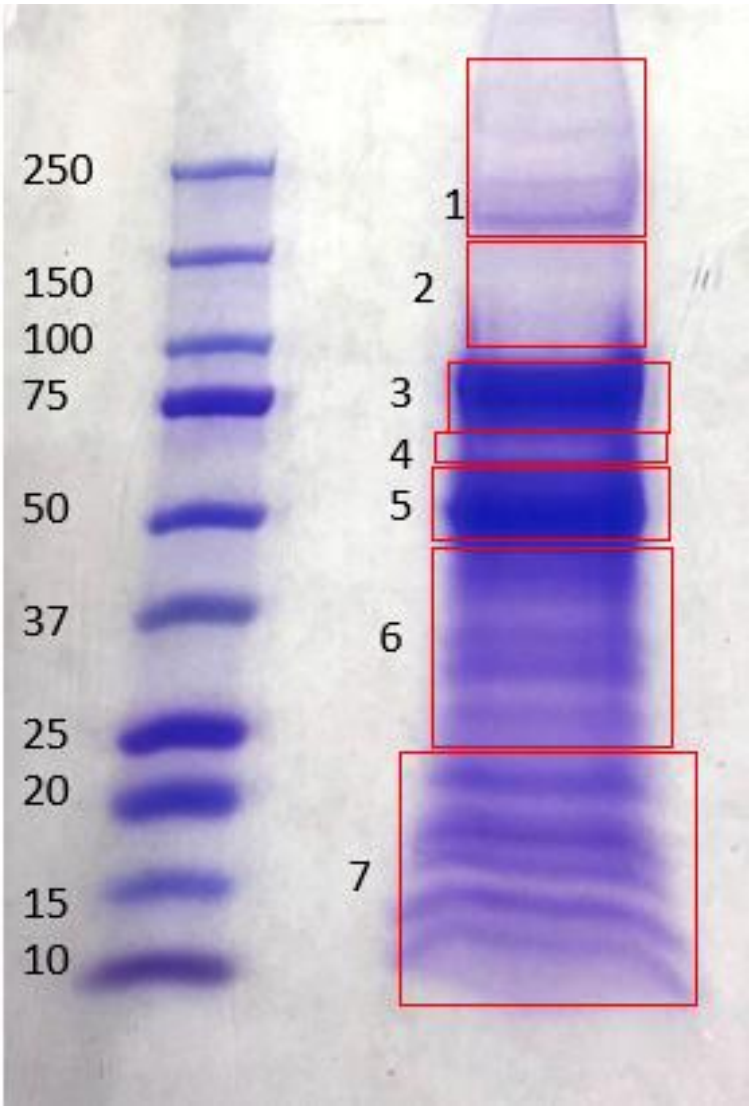
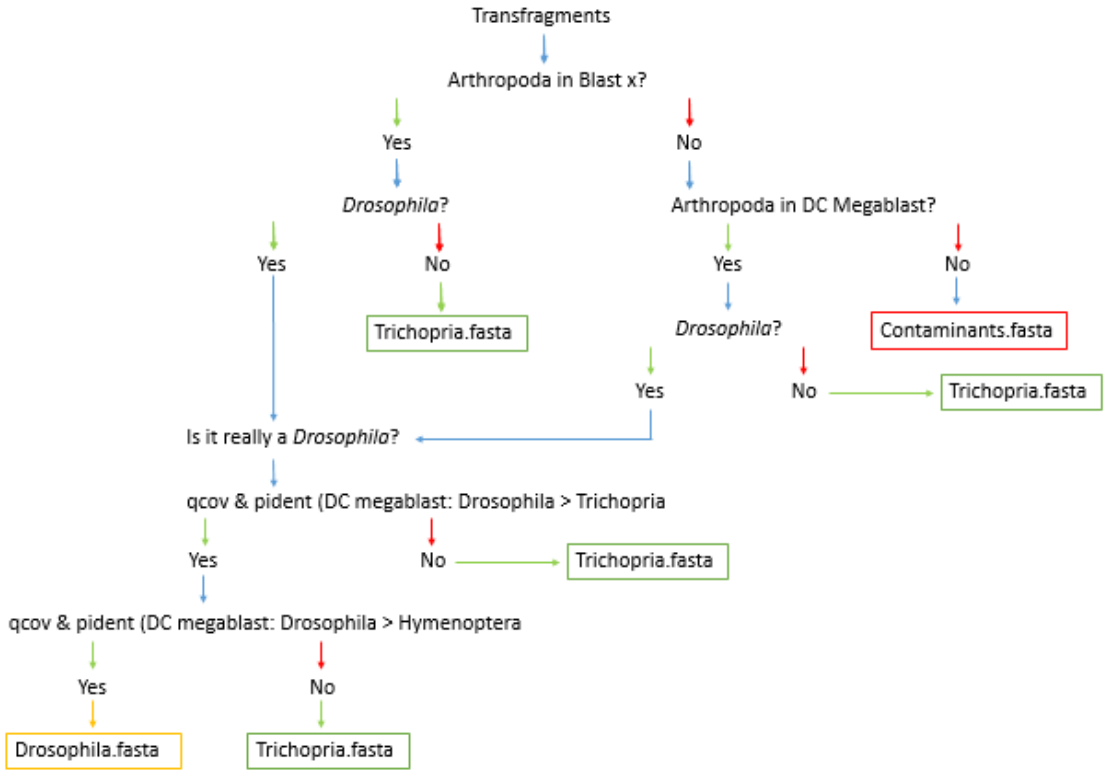
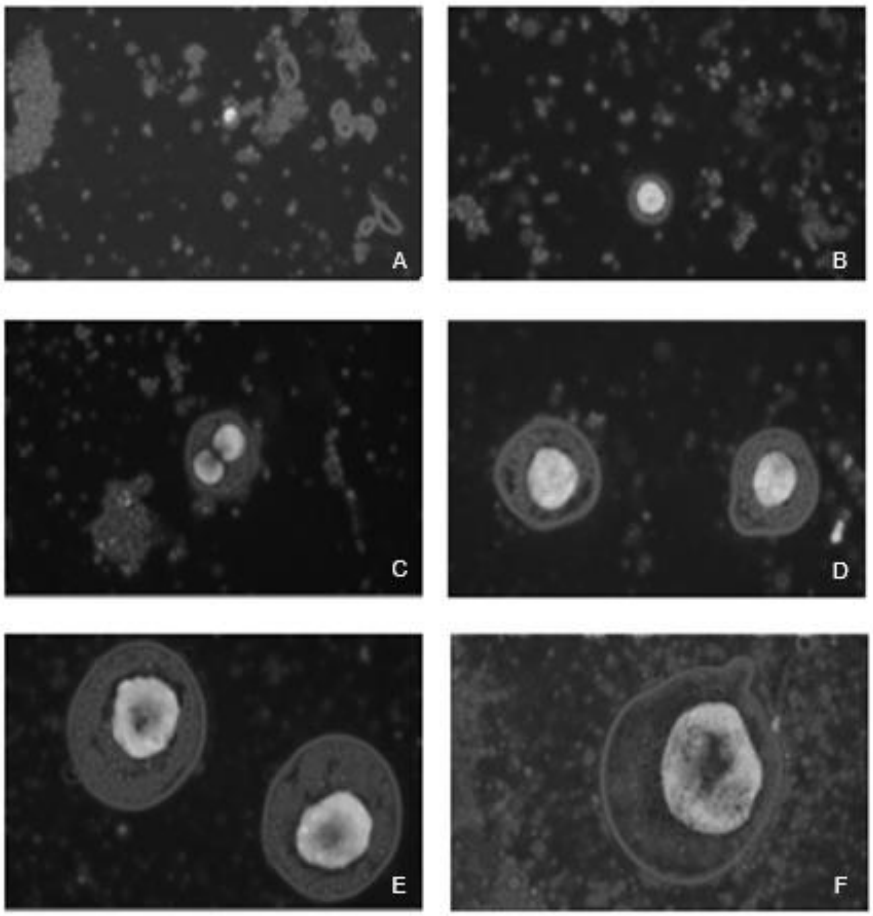


Figure S1. SDS–PAGE analysis of crude venom from *Trichopria drosophilae*.



544

545 Figure S2. Scheme of the filter build to separate the transfragments of *Trichopria drosophilae*
546 from *Drosophila* melanogaster.



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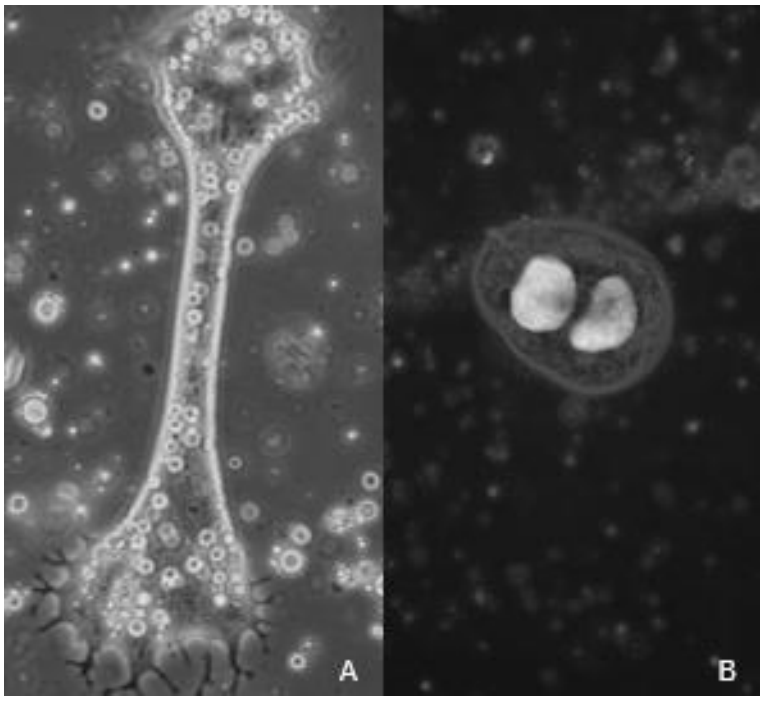
548 Figure S3. Teratocytes of *Trichopria drosophilae* at 2 (A), 3 (B), 4 (C), 5 (D), 6 (E) and 7 (F)
549 days after parasitism.



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551 Figure S4. Malformation (A) and binucleated (B) teratocytes of *Trichopria drosophilae*

Artigo 2 - BioControl

3- Artigo 2 – Mating strategies of *Trichopria anastrephae*, a parasitoid of *Drosophila suzukii*

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Abstract

Hymenopteran parasitoids, as any other insect, employee a set of strategies to ensure their reproduction. Here, we present mating strategies used by the pupal parasitoid *Trichopria anastrephae,* a potential biocontrol agent of the invasive pest species *Drosophila suzukii.* Adults from this species emerge in the first hours of photophase, and males emerge in the first days, demonstrating that *T. anastrephae* is a protandrous species. Parasitoid age when first mated influences the parasitism and sex ratio. Younger females result in a higher number of offspring, while older males result in a higher sex ratio of the offspring. Both males and females are polygamic, and the order in which a female is mated by a polygynyc male affects parasitism, viability of parasitized pupae and sex ratio of offspring, with the first female performing the highest parasitism and sex ratio, but the lowest viability. On the other hand, females that are given the opportunity to mate multiple times generate lower numbers of offspring, when compared to virgin or single mated females, but the highest sex ratio. Data present in this study can be used to improve parasitoid rearing and field releases of *T. anastrephae,* to be used in biological control programs for *D. suzukii*.

Key words: protandry, polygamy, offspring sex ratio, parasitism

Statements and Declarations

The authors declare no competing interests.

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Introduction

Insects may use sexually or asexually modes of reproduction, ensuring their multiplication (O'Woma et al. 2016). In Hymenoptera, individuals are generally haplodiploid and arrhenotoky is the dominant pattern of sex determination (Heimpel; de Boer 2008). Thus, fertilized eggs develop as diploid females and unfertilized eggs develop as haploid males. To ensure their reproduction, and the production of diploid females, hymenopteran wasps follow a sequence of events and adopt strategies, specially related to mating, that increase their genetic success (Thornhill and Alcock 1983).

One strategy frequently observed in parasitoids is protandry, a phenomenon of males emerging hours or days before females (Teder et al. 2021), that typically occurs in quasi-gregarious (solitary species that develop in hosts that are aggregated) and gregarious species (Bourdais and Hence 2019). In species that females are receptive soon after emergence and sib matings do not have deleterious effects, protandry allows males to maximize their mating opportunities (Bourdais and Hance 2019). In addition, protandry may also reduce the time between female emergence and mating event, and, in cases where copula occurs in the emergence site, it increases the chance of occurrence of mating prior to female dispersal (Pompanon et al. 1995).

Another reproductive strategy is based on the number of matings that both sexes perform in a given period (Thornhill and Alcock 1983). In most parasitoid species, female mate only once (monoandry), while males mate more than once (polygyny) (Makatiani et al. 2013). The experience gained after repeated matings, allow males of some hymenopteran wasps to respond and court more successfully with females (Perez-Lachaud and Campan 1995; He and Wang 2008). However, females that mate with experienced males may receive fewer sperm, and then, produce a higher proportion of male offspring (Steiner et al. 2008; Kant et al. 2012). Moreover, the act of mating and the number of matings may influence

(positively or negatively) female sexual receptivity, female longevity, egg maturation, fecundity and fertility (Arnqvist and Nilsson 2000). To avoid or minimize any negative consequences of mating, females of some species may mate with different males (polyandry) (Makatiani et al. 2013). Although monoandry is most common for parasitoid species (Ridley 1993), there is a tendency for gregarious and quasi-gregarious species to be polyandrous (Godfray 1994; Santolamazza-Carbone and Pestaña, 2010).

The success of mating strategies in parasitoids is often perceived in the sex ratio of the offspring, since unsuccessful matings or insufficient sperm transferred during mating will result in male biased progeny in arrhenotokous species. Thus, studying mating strategies used by parasitoids are important to improve laboratory mass rearing and increase the success of field releases (Makatiani et al. 2013), since female biased offspring is favorable for biological control due to increased population growth rates and female contribution to pest mortality (Heimpel and Lundgreen, 2000; Ramadan; Wang 2021). Here, we determined mating strategies used by *Trichopria anastrephae* Costa Lima (Hymenoptera: Diapriidae), a potential biological control agent against the invasive pest species *Drosophila suzukii* Matsumura (Diptera: Drosophilidae).

Drosophila suzukii, also known as spotted-wing Drosophila, is native from Asia and expanded its range in recent years and became a major pest of small fruits causing an economic impact mainly in countries in Europe and North and South America (dos Santos et al. 2017; Knapp et al. 2021), but its presence was also recently recorded in Africa (Boughdad et al. 2020, Hassani et al. 2020) This pest is usually controlled with insecticides (Beers et al. 2011; Bruck et al. 2011; Gress and Zalom 2019; Van Timmeren et al. 2019), but due to risks of insecticide resistance and residue limits (Diepenbrock et al. 2016; Gress and Zalom 2019), alternative control methods, such as biological control, are encouraged.

Since its first association to *D. suzukii* in southern Brazil (Wollmann et al. 2016), the quasi-gregarious, resident pupal parasitoid *T. anastrephae* has been more extensively studied and presented itself as an efficient parasitoid of *D. suzukii* in different laboratory conditions (Krüger et al. 2019; Vieira et al. 2019; 2020, Garcia, 2020a, Tait et al. 2021). Nowadays, *T. anastrephae* is considered one of the parasitoids of the Neotropical Region with the greatest potential to be used in future biological pest control programs (Garcia 2020b, Garcia et al., 2022). This parasitoid also shows potential to search and find hosts located in attacked fruits (De la Vega et al. 2021), and successfully parasitize *D. suzukii* in the presence of other parasitoid (Oliveira et al. 2021) or other products such as insecticides used to control the fly (Geisler et al. 2019; Schlesener et al. 2019, Shawer, 2020, Tait et al. 2021). However, information is lacking on the reproductive strategies employed by *T. anastrephae*, and their relationship to parasitism ability. Thus, in the present study we conducted a series of experiments to investigate the linkage between mating strategies and parasitism aspects, using *D. suzukii* as host.

Material and methods

Maintenance rearing of *D. suzukii* and *T. anastrephae*

Rearing of *D. suzukii* and *T. anastrephae*, as well as all experiments here described, were conducted under controlled conditions (23 ± 2 °C, 70 ± 10 % r.h. and 12 h of light). Both colonies originated from infested fruits collected in nearby organic farms, and new insects were periodically introduced to maintain colony vigor. Flies were reared on a cornmeal-based diet (described by Schlesener et al. 2018), and adults were held in plastic cages (262 × 177 × 147 mm) (length × height × width), with two side openings (80 × 100 mm), and an opening in the lid (155 × 50 mm), covered with voile cloth. Parasitoids were

reared on *D. suzukii* pupae, and adults were held in plastic cages, as described previously, and fed pure honey (ad libitum).

Emergence pattern

Pupae of *D. suzukii* (less than 12 h old) were exposed to *T. anastrephae* females (15 pupae/female), for a total of 30 females. Groups of 15 pupae were placed in plastic lids (20 mm diameter), containing humid cotton balls, and exposed to parasitoid females for 6 hours. Then, pupae were individualized in micro tubes (1.5 mL), and 14 after exposure to parasitism, emergence of pupae were verified every two hours during photophase (7h00 to 19h00). In each observation, number and sex of parasitoids were verified. Observations occurred until there was no parasitoid emergence for two days.

To verify if the age of males and females have influence on parasitism and sex ratio (proportion of female) of the offspring, we exposed *D. suzukii* pupae to *T. anastrephae*, as described before. To obtain enough parasitoids from different ages, different batches of pupae were exposed to parasitoids during three consecutive days, and each batch were exposed for 24 h. After exposure, parasitoids were individually placed in micro tubes (1.5 mL), and at the moment of emergence they were coupled according to the treatments. The treatments were formed by 1, 2 or 3 d old males coupled with 1, 2 or 3 d old females, in every possible combination, resulting in 9 treatments. Couples, according to each treatment, were placed in plastic cups (200mL), with a hole (15 mm diameter) on top, covered with voile fabric. Pure honey and water were provided in plastic lids (10 mm diameter) containing cotton.

During 14 days, 15 *D. suzukii* pupae were offered daily for each couple. After exposure period, pupae were removed and placed in acrylic tubes (25 × 45 mm). After the emergence of flies and parasitoids, parasitoids were sexed and counted, and the pupae that remained intact were dissected, and the presence of flies or parasitoids to determine actual

parasitism rates. Percentage of parasitized pupae [(number of emerged + non-emerged parasitoids)/15] per female, viability of parasitized pupae [(number of emerged parasitoids*100)/number of parasitized pupae], number of emerged parasitoids and sex ratio were evaluated. The experimental design was completely randomized with 20 replicates per treatment.

Polygyny

After emergence, virgin males (obtained as described before) were individualized in plastic cups (200 mL). A virgin female was added to each male, and the occurrence of mating was observed. After mating ended, the female was removed, and a new virgin female was added to each male, until the male had mated with ten females. Matings were observed during the photophase (7h00 – 19h00). After removal, mated females were individualized in plastic cups (200 mL), and identified according to the male which they mated and the order in which they were mated. Females were fed pure honey and water, and during 14 days, 15 *D. suzukii* pupae were offered daily. After exposure, pupae were removed and kept in acrylic tubes for emergence of flies and parasitoids. In this experiment we evaluated the number of females mated by each male, percentage of parasitized pupae, viability of parasitized pupae and number and sex ratio of emerged parasitoids. The experimental design was completely randomized with 20 replicates per treatment (order in which females were mated).

Polyandry

Newly emerged virgin females were individualized in plastic cups (200 mL), and two virgin males were placed with each female. Occurrence of mating with one or both males were observed, during the first four hours of photophase (7h00 – 11h00). After observation period, both males were removed. Virgin males were offered every three days for 12 days,

totaling 5 opportunities to mate. During the entire period, females were fed pure honey and water, and 15 *D. suzukii* pupae were offered daily. After exposure, pupae were placed in acrylic tubes for emergence, and percentage of parasitized pupae, viability of parasitized pupae and number and sex ratio of emerged parasitoids were evaluated. The experimental design was completely randomized with 20 replicates (females).

Effects of mating on female longevity and parasitism

To obtain virgin parasitoids for mating experiments, *D. suzukii* pupae were exposed to *T. anastrephae*, and after 24 h of exposure, they were individualized in micro tubes, as described before. Soon after emergence, females were individually placed in plastic cups with pure honey and water (as previously described). The treatments were virgin females (no males were placed in the plastic cups), single mated females (females were allowed to mate once, and male was removed soon after the mating couple unplugged) and multiple mated females (males were kept with female during entire lifespan of the female). The experimental design was completely randomized with 20 replicates per treatment.

For parasitism, 15 *D. suzukii* pupae were offered daily during the entire lifespan of the female. After exposure, pupae were removed, placed in acrylic tubes, and after emergence, parasitoids were sexed and counted and intact pupae were dissected to determine actual parasitism rates. Percentage of parasitized pupae, viability of parasitized pupae, number of emerged parasitoids and sex ratio were evaluated.

Statistical analysis

Number of emerged parasitoids according to period of the day were compared using general linear models (GLM) with a quasibinomial error distribution ($p \leq 0.05$). Generalized linear models (GLM) with a quasibinomial distribution was also used to assess the effects of

female age and male age (i.e. 1, 2 or 3 d old), mating (i.e. virgin, single mated or multiple mated females) and polygyny on percentage of parasitism, viability of parasitized pupae, number of offspring and sex ratio of *T. anastrephae*. In case of significance, Tukey test ($p \leq 0.05$) was applied for multiple comparison. To verify the occurrence of protandry in the emergence pattern bioassay, a chi-squared test ($p \leq 0.05$) was used to compare the frequency of males and females emerged in different intervals of egg-to-adult period. Longevity of virgin, single and multiple mated females was subjected to survival analysis using Kaplan–Meier estimator, and compared using log-rank test. All statistical analyses were performed using R software version 4.0.3 (R Core Team, 2020).

Results

Emergence pattern

From 450 *D. suzukii* exposed to *T. anastrephae* females, 249 resulted in the emergence of parasitoids. Emergence presented a peak in the first two hours of photophase (07:01-09:00), when 51.41% of parasitoids emerged, and decreased along the day ($\chi 2 = 289.43$, $p < 0.0001$; Figure 1a). The emergence pattern was also different according to sex ($\chi 2 = 197.85$, $p < 0.0001$; Figure 1b). Most males (94.19%) emerged 17 to 21 days after parasitism, while the emergence of females started later (first females emerging 18 days after parasitism), and it was more evenly distributed from 19 to 27 days after parasitism.

Influence of parasitoid age on parasitism and sex ratio

The female age affected the number of offspring, while the male age affected the sex ratio of the offspring (Table 1). The higher number of offspring was obtained when females were 1 d old (8.16 ± 0.15), differing from 2 and 3 d old females (7.74 ± 0.14 and 7.70 ± 0.10, respectively), regardless of male age. The higher sex ratio was obtained when females

(regardless of age) were mated with males were 3 d old (0.64 ± 0.02), differing from 1 d old males (0.56 ± 0.03). Meanwhile, the sex ratio of the offspring from females mated with 2 d old males (0.60 ± 0.02) did not differ from the sex ratio of offspring from females mated with either 1 nor 3 d old males.

Occurrence of polygyny and effects on parasitism

All males of *T. anastrephae* tested were able to mate with all 10 females provided. The order in which a female was mated affected the percentage of parasitism ($\chi 2 = 14.14$, $p = 0.0169$; Table 2), viability of parasitized pupae ($\chi 2 = 3.43$, $p = 0.0262$; Table 2) and sex ratio of offspring ($\chi 2 = 5.93$, $p < 0.0001$; Table 2), but did not affect the number of offspring ($\chi 2 = 1.06$, $p = 0.4895$; Table 2). The first female mated showed the highest percentage of parasitism (63.83 ± 0.78%) and generated the offspring with the highest sex ratio (0.61 ± 0.05), but resulted in the lowest viability of parasitized pupae (92.92 ± 0.57%).

Occurrence of polyandry and effects on parasitism

All tested females mated during the 5 opportunities they were given. Even after multiple matings, the percentage of parasitism and female offspring still decreased during the 12 days of observation (Figure 2). On the other hand, percentage of viability presented a sharp decrease in the 8^{th} and 9^{th} day after the first mating, but showed an increase towards the 12^{th} day (Figure 2).

Effects of mating on female longevity and parasitism

Mating did not affect the longevity of females ($\chi 2 = 4.50$, $p = 0.1000$). Virgin females lived 29.90 ± 1.67 days, while single and multiple mated females lived for 35.20 ± 1.46 and 29.00 ± 2.33 days, respectively. Moreover, mating affected the number and sex ratio of offspring ($\chi 2 = 44.12$, $p = 0.0107$ and $\chi 2 = 13.96$, $p < 0.0001$, respectively; Table 3), but did

not affect percentage of parasitism and viability of parasitized pupae ($\chi 2 = 15.83$, $p = 0.0964$ and $\chi 2 = 0.04$, $p = 0.9239$, respectively; Table 3). The highest number of offspring was obtained from virgin females (135.20 ± 2.89), not differing from single mated females (130.70 ± 6.86), whereas the lowest number of offspring were obtained from multiple mated females (113.05 ± 5.90). On the other hand, the highest sex ratio of offspring was obtained from multiple mated females (0.49 ± 0.04), whilst, not surprisingly, virgin females were unable to produce female descendants.

Discussion

To ensure their successful reproduction, insects adopt distinct strategies. In this study we showed some of the strategies, related to mating and parasitism, used by *T. anastrephae*, a pupal parasitoid resident from South America (Cruz et al, 2011), and a potential biocontrol agent for the management of *D. suzukii* (Garcia 2020b). First, we showed that these parasitoids, in laboratory, emerge in the first hours of photophase, and males emerge few days prior than females. The age of females and males impact the number and sex ratio of offspring. Then, we showed that both males and females are polygamic, in laboratory, and the order in which a female is mated by a male affects parasistism, viability of parasitized pupae and sex ratio of the offspring. We also showed that mating per se also affects the number and sex ratio of offspring.

Regarding the emergence pattern of *T. anastrephae*, we observed many similarities of this species with other parasitoids, including a peak following the lighting onset and protandry (Zaslavski et al. 1999; Sakai and Ishida 2001; Doyon and Boivin 2005; Bourdais and Hance 2019). The emergence in the first few hours of photophase is probably mediated by photoreceptors and aclosion hormone activation (Bourdais and Hance 2019). Emergence during the first hours of light also coincides with more favorable conditions in nature, as

261 milder temperatures and higher humidity, reducing risks of water loss of newly emerged
262 insects (Lankinen 1986). Also, emergence after the first hours of daylight has been suggested
263 to favor reproductive behavior in parasitoids (Pompanon et al. 1995; Karpova and Reznik
264 2002; Karpova 2006).

265 Another point regarding emergence pattern is that *T. anastrephae* males usually
266 emerge before females, but with overlaps, reducing the protandrous status of this species.
267 Other species showing a similar pattern is *Aphidius matricariae* (Haliday) (Hymenoptera:
268 Braconidae), *Aphidius ervi* Haliday (Hymenoptera: Braconidae) and *Cotesia glomerata*
269 (Linnaeus) (Hymenoptera: Braconidae) (He et al. 2004; Mazzi et al. 2011; Bourdais and
270 Hance 2019). As a quasi-gregarious species, the overlap in the emergence of males and
271 females may facilitate inbreeding. However, to our knowledge, the costs of inbreeding has not
272 yet been studied for this species, although sib mating is known to have deleterious effects on
273 some hymenopteran species (Heimpel and de Boer 2008). According to our observations,
274 newly emerged males tend to stay closer to the same cluster where they emerge, instead of
275 dispersing, and they mate with newly emerged females as soon as they leave the host pupal
276 case, indicating that sib mating may be a common behavior in this species. This behavior of
277 local mating could reduce genetic diversity, but also reduce potential costs of dispersal.

278 Since males tend to emerge before than females, but there is still some overlap, we
279 tested whether age of the parents would affect the progeny. We found that younger females,
280 regardless of the age of the male they mated, produced a larger offspring. This phenomenon
281 may be consequence of host deprivation, since females mated when 2 or 3 days old did not
282 receive any host prior to mating. It has been shown before that when *T. anastrephae* is host-
283 deprived for 3 days, they produce less offspring than those that were able to parasitize soon
284 after emergence (Krüger et al. 2019). Meanwhile, females, regardless of their age, mated with
285 older males, produced an offspring with higher percentage of females. Since all males used in

the experiment, regardless of age were virgin, experience is rule out as a reason why mating with older males resulted in an offspring with a higher sex ratio. The effects of paternal age on the progeny is poorly studied; however, it is possible that older males were able to transfer higher amounts of sperm at the moment of mating, or the sperm viability increases when males age (García-González and Simmons 2005).

When males are given the chance to mate with several females, first mated females produce an offspring with a higher sex ratio compared to subsequently mated ones. This is consistent with results obtained for other parasitoids (Kant et al. 2012; Makatiani et al. 2013), and suggests a decrease in sperm transferred after each copulation (Damiens and Boivin 2005). In our experiment, females were replaced right after successful mating by a virgin female, encouraging males to successively mate in a short period of time. However, males of some species of parasitoids are able to replenish their sperm when given an interval between copulations (synspermatogenic), whereas, some males could get permanently sperm depleted (prospermatogenic) (Boivin et al. 2005).

Although *Trichopria* spp. are expected to be monoandrous (Carton et al. 1986), when given the chance, all *T. anastrephae* females tested accepted several matings. However, laboratory strains may present a higher tendency to re-mate (Burton Chellew et al. 2007) to avoid male harassment (convenience polyandry) or to replenish their spermathecal (Santolamazza-Carbone and Pestaña, 2010). In the field, some parasitoid species usually mate only once upon emergence, before dispersing (Tooke 1955), but polyandry level of *T. anastrephae* in the field was not yet estimated. It is important to note that even parasitism and sex ratio of offspring produced by polyandryc females show a decreasing pattern with time after first mating, and were not recovered by subsequent matings.

A previous study reported that multiple matings may increase the longevity of females of an egg parasitoid (Jacob and Boivin 2005). However, in our experiment mating

311 status/frequency did not affect female lifespan, suggesting mating presents a low mating cost
312 for this species (Jacob and Evans 2000; Santolamazza-Carbone and Pestaña 2010). Although
313 some species may transfer nutritional resources during mating (Santolamazza-Carbone and
314 Pestaña 2010), in our experiments mated and unmated females were provided with
315 carbohydrates and hosts during the entire experiment, and the nutritional provision may also
316 have an impact on longevity.

317 Mating also could influence parasitism behavior of females. Virgin females produced
318 higher number of offspring, which was comparable to single mated females, while multiple
319 mated females produced the lowest. An increase in egg-laying activity in unmated females
320 was previously reported in *Anagrus delicatus* Dozier (Hymenoptera: Mymaridae),
321 *Bathyplectes curculionis* (Thomson) (Hymenoptera: Ichneumonidae) and *Habrobracon*
322 *hebetor* Say (Hymenoptera: Braconidae) (Cronin and Strong 1990; Jacob and Evans 2000;
323 Seyahooei et al. 2018). It has been suggested that producing female progeny results in a
324 higher energy cost to the female then producing male progeny (Seyahooei et al. 2018), thus,
325 virgin females, that only produce sons, have a spend lower energy and can produce more
326 offspring.

327 As arrhenotoky is the dominant pattern of sex determination in Hymenoptera
328 (Heimpel and de Boer 2008), it was not surprisingly that virgin females produced male
329 offspring only. When kept with a male counterpart during its lifetime, *T. anastrephae* female
330 produced an overall higher proportion of females than females mated only once. The increase
331 in the sex ratio of the offspring when the female mate more than once was showed previously
332 for other species of parasitoids (Chevrier and Bressac 2002; Sagarra et al. 2002). Multiple
333 mating may help with both quality and quantity of sperm received from males, and positively
334 impact offspring production and sex ratio (Hegazi et al. 2020). For mass rearing purposes, an

abundance of males should be provided to females, so they could remate as much as needed, resulting in plenty availability of sperm used in sex allocation.

The results presented in these studies give new insights about the relationships of emergence pattern, protandry, polygamy and mating status on parasitism and progeny viability and sex ratio. Information regarding reproduction strategies is important to be considered for ecological purposes and in mass rearing of parasitoids for augmentative release in biological control. However, mating systems of *T. anastrephae* remain poorly understood and future studies would benefit by considering costs of inbreeding and the possibilities of manipulating parasitoid rearing in order to obtain higher sex ratios, since it would increase the chances of success of this species in a biological control program against *D. suzukii*.

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558

559 **Figure captions**
560
561 **Figure 1** Emergence rhythm of *Trichopria anastrephae* along the day (a) and egg to adult
562 period of males and females of *Trichopria anastrephae* (b) Different lower case letters letters
563 indicate significant differences among treatments according to Tukey test (p≤ 005).
564 **Figure 2** Percentage of parasitism, viability of parasitized pupae and female offspring produced
565 by polyandryc *Trichopria anastrephae* females Bars present standard error.

Table 1: Generalized linear models (GLM) with a quasipoisson error distribution to evaluate the influence of parasitoid age (female and male) on percentage of parasitized pupae, number of offspring, viability of parasitized pupae and sex ratio of *Trichopria anastrephae*

Dependent variables	Interaction female × male		Female		Male	
	χ^2	p	χ^2	p	χ^2	p
Percentage of parasitism	11.99	0.6958	14.20	0.2690	3.37	0.7322
Number of Offspring	0.91	0.1081	0.84	0.0306	0.14	0.5582
Viability of parasitized pupae	0.56	0.8188	0.05	0.9354	0.01	0.9851
Sex ratio	0.04	0.9200	0.25	0.0752	0.29	0.0451

Table 2 Average (± se) percentage of parasitism, viability of parasitized pupae (%), total number of offspring and sex ratio of offspring produced by females of *Trichopria anastrephae* mated with polygynic males

Order of mating	Percentage of parasitism [α]	Viability of parasitized pupae (%)[α]	Number of offspring [ns]	Sex ratio [α]
1º	63.83 ± 0.78 a	92.92 ± 0.57 b	8.81 ± 0.11	0.61 ± 0.05 a
2º	59.02 ± 1.99 ab	97.39 ± 0.62 a	8.63 ± 0.31	0.54 ± 0.04 ab
3º	60.86 ± 1.12 ab	94.70 ± 2.11 ab	8.70 ± 0.28	0.36 ± 0.05 bc
4º	61.53 ± 0.70 ab	96.60 ± 0.42 ab	8.94 ± 0.10	0.36 ± 0.04 bc
5º	59.35 ± 2.66 ab	95.21 ± 1.16 ab	8.56 ± 0.39	0.29 ± 0.03 c
6º	58.90 ± 1.16 ab	96.38 ± 0.80 ab	8.57 ± 0.19	0.28 ± 0.03 c
7º	59.60 ± 1.08 ab	96.71 ± 0.42 ab	8.67 ± 0.17	0.28 ± 0.04 c
8º	58.38 ± 1.06 ab	97.08 ± 0.49 a	8.51 ± 0.17	0.28 ± 0.04 c
9º	58.97 ± 1.15 ab	96.68 ± 0.68 ab	8.59 ± 0.19	0.33 ± 0.05 b
10º	55.41 ± 1.90 b	96.59 ± 0.63 ab	8.06 ± 0.29	0.30 ± 0.04 c

[ns] not significant

[α] Different lowercase letters indicate significant differences among treatments according to Tukey test (p≤ 005)

Table 3 Average (± se) percentage of parasitism, viability of parasitized pupae (%), total number of offspring and sex ratio of offspring produced by virgin, single and multiple mated females of *Trichopria anastrephae*

Treatments	Percentage of parasitism [ns]	Viability of parasitized pupae (%) [ns]	Number of offspring [α]	Sex ratio [α]
Virgin	34.73 ± 2.43	92.06 ± 1.09	135.20 ± 2.89 a	0.00 ± 0.00 c
Single mated	27.76 ± 1.80	91.69 ± 1.04	130.70 ± 6.86 ab	0.34 ± 0.04 b
Multiple mated	32.05 ± 2.66	92.30 ± 1.13	113.05 ± 5.90 b	0.49 ± 0.04 a

[ns] not significant

[α] Different lowercase letters indicate significant differences among treatments according to Tukey test (p≤ 005)

Figure 1:

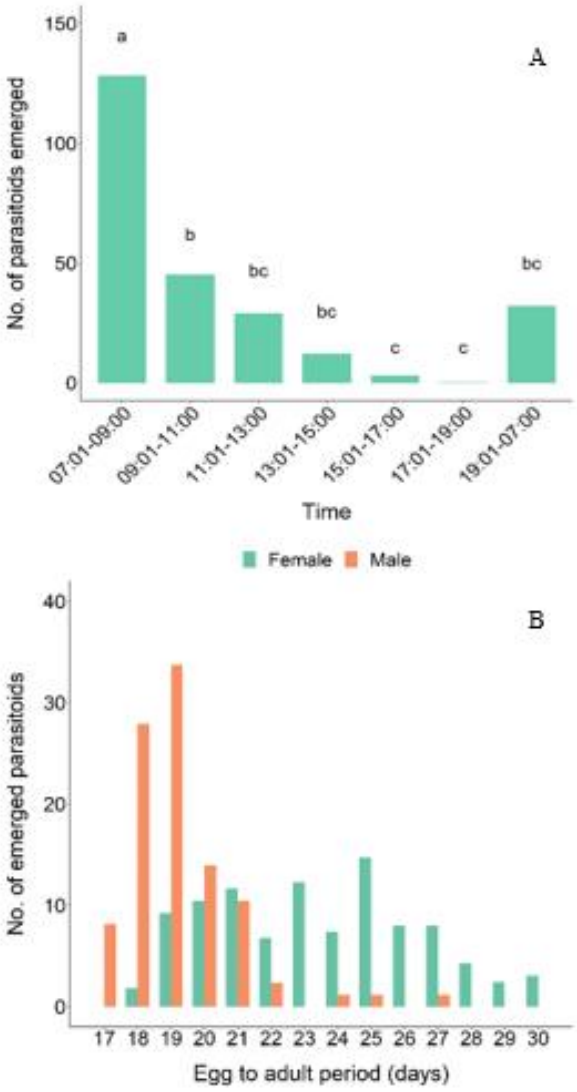
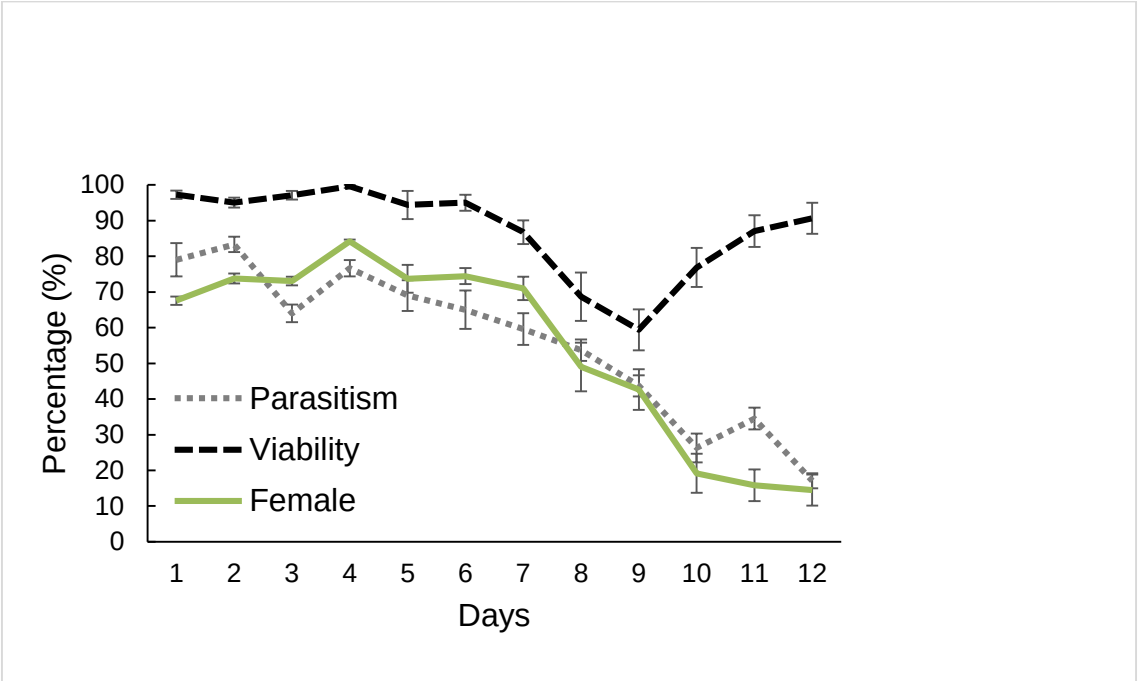

A
No. of parasitoids emerged
150
100
50
0
a
b
bc
bc
c
c
bc
07:01-09:00
09:01-11:00
11:01-13:00
13:01-15:00
15:01-17:00
17:01-19:00
19:01-07:00
Time
Female
Male
B
No. of emerged parasitoids
40
30
20
10
0
17 18 19 20 21 22 23 24 25 26 27 28 29 30
Egg to adult period (days)

Figure 2:


100
90
80
70
60
50
40
30
20
10
0
Percentage (%)
Parasitism
Viability
Female
1
2
3
4
5
6
7
8
9
10
11
12
Days

Artigo 3 – Neotropical Entomology (published)

4- Artigo 3 - Effects of extrinsic, intraspecific competition and host deprivation on the biology of *Trichopria anastrephae* (Hymenoptera: Diapriidae) reared on *Drosophila suzukii* (Diptera: Drosophilidae)

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Running title: Intraspecific competition and host deprivation effects on *Trichopria anastrephae*

Abstract

The pupal parasitoid *Trichopria anastrephae* Lima (Hymenoptera: Diapriidae) shows potential to control *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), and understanding the behavior of this parasitoid in challenging environments is important to obtain a higher efficiency in mass-rearing and in biological control programs. This study aimed to verify the effects of extrinsic intraspecific competition and the absence of host on the parasitism of *T. anastrephae* in *D. suzukii* pupae. Therefore, to evaluate the parasitism of *T. anastrephae* under intraspecific competition, groups of 20 pupae (24 h old) of *D. suzukii* were offered for different densities of parasitoids (1, 3, 5 or 10 couples) during a seven days period. Whereas to evaluate the effects of host deprivation on parasitism of *T. anastrephae*, we tested different treatments: T1- No deprivation, T2- Complete deprivation, T3- Deprivation every other day, T4- Deprivation for 3 days, and T5- Deprivation for 7 days. The increase of density of parasitoids resulted in an increase of oviposition scars on pupae and a longer biological cycle, suggesting the occurrence of superparasitism. Increased density also resulted in a higher percentage of attacked pupae, but did not affect parasitoids emergence nor sex ratio. Host deprivation affected number of parasitized pupae, number and sex ratio of offspring, and the longevity of females. Based on our findings, competition among females do not impair offspring viability, and host deprivation for a period up to seven days do not influence parasitism capacity, indicating that it can be used as a pre-release strategy.

Keywords: Biological control, pupal parasitoid, spotted-wing drosophila, SWD, parasitoid-host interaction, sex ratio, longevity, viability

Introduction

The spotted-wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is native to Asia, but it is currently distributed in Europe, North and South America (Bolda *et al* 2010, Walsh *et al* 2011, Calabria *et al* 2012, Cini *et al* 2012, Deprá *et al* 2014) and has potential to invade and establish in other continents (Dos Santos *et al* 2017). Due to its highly sclerotized ovipositor, the females are able to lay eggs in ripening and mature healthy fruits, where the larvae develop, hindering the commercialization of fresh fruits and resulting in economic losses (Schlesener *et al* 2015).

Drosophila suzukii management is based mainly on chemical products (Bruck *et al* 2011, Burrack *et al* 2015). However, the short life cycle and the limited residual control provided by insecticides induce the frequent use of products to keep the population density low (Renkema *et al* 2016). In addition, the first case of resistance to insecticide by a strain of *D. suzukii* was recently reported (Gress & Zalom 2018), showing the urge to adopt an integrated management for this pest, encouraging the use of a variety of methods and tactics of control, such as biological control.

Studies performed in Europe and North America reported the association of *D. suzukii* with a variety of parasitoid species, although only few species showed efficiency in population growth regulation (Chabert *et al* 2012, Rossi Stacconi *et al* 2013, Gabarra *et al* 2015, Rossi Stacconi *et al* 2015, Garcia *et al* 2017). Due to the elevated immune system of *D. suzukii* larvae, pupal parasitoids, such as *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae) and *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) seem to be more successful against this pest (Kacsoh & Schlenke 2012). The pupal endoparasitoid *Trichopria anastrephae* Lima (Hymenoptera: Diapriidae), native from South America (Garcia & Corseuil 2004, Cruz *et al* 2011), was found in fruits infested by *D. suzukii* in Brazil, and showed potential to parasitize the pest in laboratory (Wollmann *et al* 2016, Andreazza *et al* 2017). Despite *T.*

anastrephae exhibit potential to be used in biological control programs to control *D. suzukii*, there is scarcity of information about this parasitoid species in the literature (Wollmann *et al* 2016, Andreazza *et al* 2017, Bernardi *et al* 2017, Schlesener *et al* 2019).

In laboratory (and mass) rearing, parasitoid adults are kept in groups, in cages or vials, and hosts are offered in a periodic basis, to produce more parasitoids (Schlesener *et al* 2019). In this environment, parasitoid females are competing for hosts, and this competitive interaction can affect viability and sex ratio of offspring, and favor the occurrence of superparasitism (Hamilton 1967, Carneiro *et al* 2009, Cruz *et al* 2018). According to the Local Mating Competition theory (LMC), proposed by Hamilton (1967), when a single female oviposits in a patch, the progeny will be female-biased, whereas in competitive environments a less female-biased offspring is expected. Consequently, the increase in male progeny can be unfavorable for mass-rearing which aim to maximize female production for colony maintenance and field release (Irvin & Hoddle 2006). Moreover, if the competitive environment is extreme, it can be detrimental when it stimulates superparasitism to the point which emergence of parasitoids is influenced (Cruz *et al* 2018).

Host deprivation is another factor that can affect the success of biological control. Before releasing in the field, usually parasitoids undergo a pre-release conditioning, where they are provided with carbohydrate sources, attempting to maximize the fitness of parasitoids (Hougardy *et al* 2005, Tena *et al* 2017). During this period, host deprivation occurs, and this can affect population dynamics and production of parasitoid offspring (Hougardy *et al* 2005). In some cases, host provision during pre-release conditioning can be positive since it can stimulate oogenesis and host searching in field (Tena *et al* 2017), while in other cases host deprivation during this period are essential to ensure sufficient fecundity and longevity post-release (Hougardy *et al* 2005). Furthermore, after field releasing, parasitoids can undergo

periods of low host availability and they may absorb oocytes, or retain eggs to compensate the host absence period with an extension in reproductive life (Carneiro *et al* 2009).

Understanding the behavior of a parasitoid in intra and interspecific interactions is important to obtain a higher efficiency in mass-rearing, improvement of quality control, and help biological control programs to become more efficient (Montoya *et al* 2003). Thus, the objective of this study was to verify the effects of intraspecific competition among females and host deprivation on the parasitism of *T. anastrephae* in pupae of *D. suzukii*.

Material and methods

Maintenance rearing of *Drosophila suzukii*

The laboratory rearing originated from flies emerged from infested blackberries (Rubus spp.) collected in January 2016, in Pelotas, Rio Grande do Sul, Brazil (31°38′20″S and 52°30′43″W). Since its establishment, the laboratory colony did not receive any introduction of wild flies, and the colony was maintained for over two years, when the experiments were conducted. The flies were reared on artificial diet in glass tubes (25 x 85 mm) capped with cotton plugs, as described by Schlesener *et al* (2017) and Schlesener *et al* (2018). The artificial diet consisted of agar (8 g), yeast (40 g), cornmeal (80 g), sugar (100 g), propionic acid (3 mL), methyl paraben (0.8 g dissolved in 8 mL of 90% ethanol), and water (1,000 mL). This diet was used as oviposition substrate by adults, as well as food for larvae and adults. All rearing and experiments were carried out in controlled conditions (Temperature: 25 ± 2°C, Relative humity: 70 ± 10% and photophase: 12 hours).

Maintenance rearing of *Trichopria anastrephae*

The laboratory colony of *T. anastrephae* was established with parasitoids found in *D. suzukii* infested blackberry collected in Pelotas, Rio Grande do Sul, Brazil (31°38′24″ S and 52°30′55″W) in summer 2017. Since its establishment, the laboratory rearing did not receive any introduction of wild parasitoids. After the emergence of the parasitoids that originated the laboratory colony, some specimens were collected for identification, and sent to Valmir A. Costa, who positively identify the specimens as *T. anastrephae*, according to the taxonomic characters established by Costa Lima (1940). Adults of *T. anastrephae* were kept in plastic cages (262 × 177 × 147 mm) (length × height × width), with two side openings (80 × 100 mm), and an opening in the lid (155 × 50 mm), covered with voile cloth. The adults were fed streaks of honey the top of the cage. Daily, 24h-old SWD pupae were placed in Petri dishes (60 mm diameter) lined with a moistened cotton layer and exposed to parasitism for 24h. Then, the Petri dishes containing the pupae were placed in another cage, with the same dimensions as mentioned above, until the emergence of new parasitoids.

Intraspecific competition

We tested four different densities (treatments): 1, 3, 5 and 10 couples of *T. anastrephae* (1 day old). The couples were kept at plastic cups (500 mL) containing a hole in the top (40 mm diameter) covered with voile fabric to allow ventilation, and a side opening (40 mm diameter) also made with voile fabric, to allow the change of the parasitism units. Each parasitism unit was composed of 20 *D. suzukii* pupae (<24h old) distributed on a plastic plate (25 mm diameter) lined with a moistened cotton layer. The insects were offered a plastic plate (25 mm diameter) containing a cotton ball soaked in honey, which was replaced every other

day. The parasitism units were changed every 24 hours, for 7 consecutive days. The experimental design was completely randomized with 12 replicates per treatment.

After each exposition of the pupae to the parasitoids, we observed the number of oviposition scars on the surface of each pupae produced by the insertion of parasitoid ovipositor in ovipositions or oviposition attempts. Then, the pupae were individualized, according to treatment, replicate and number of scars, in microtubes (2.0 mL). Daily, the pupae were observed to verify the duration of the biological cycle (egg-adult period) of offspring. After the emergence, the parasitoids were separated by sex to determine sex ratio (sr = number of females / number of females + males). The percentage of attack was calculated based on the total number of pupae with oviposition scars divided by the total number of offered pupae, and the viability was calculated from the number of emerged parasitoids divided by the number of pupae containing scars.

Host deprivation

Couples of *T. anastrephae* (1 day old) were placed in plastic cups (200 mL), with a hole (15 mm diameter) on top, covered with voile fabric. Honey was provided to the parasitoids as food source, as described previously. The following treatments were tested: T1) hosts provided daily, until the death of the female, T2) complete host deprivation during the entire life of the female, T3) provision of hosts in alternated days, T4) host deprivation during the first three days, and T5) host deprivation during the first seven days. In treatments T4 and T5, after the host deprivation period, host was provided daily during the entire life of the female. As host, 20 pupae of *D. suzukii* were exposed to each couple in parasitism units as described before, for a period of 24 hours. After exposure period, pupae were removed and placed in acrylic tubes (25 × 45 mm).

Daily the tubes containing the exposed pupae were observed to verify the emergence of parasitoids and, thus, determine the biological cycle of offspring. After emergence, parasitoids were sexed and counted. In addition, the flies that emerged from the pupae exposed to each treatment were counted, and the pupae that remained intact were dissected, and the presence of flies or parasitoids was recorded to determine the actual parasitism rates. Longevity of males and females, number of parasitized pupae (number of emerged + non-emerged parasitoids) per female, number of offspring generated per female and their sex ratio were evaluated. The experimental design was completely randomized with 15 replicates per treatment.

Statistical analysis

All data were verified for normality and the homoscedasticity of the residues through the Shapiro Wilk and Bartlett tests, respectively. Data from the intraspecific competition bioassay were evaluated using regression models ($p \leq 0.05$). Total number of parasitized pupae and sex ratio data from the host deprivation bioassay were submitted to analysis of variance ($p \leq 0.05$), and when confirmed statistical significance data was compared using Duncan test ($p \leq 0.05$). Total number of offspring and duration of biological cycle data from the host deprivation bioassay did not meet the normality and homoscedasticity assumptions, and were therefore submitted to the Kruskal-Wallis test ($p \leq 0.05$), and when confirmed statistical significance, data was compared by the Dunn's test ($p \leq 0.05$). The longevity of parasitoids was evaluated using the survival curves in the Kaplan-Meier estimator, and subsequently compared to each other using the log-rank test. All statistical analyzes were performed in R software (R Development Core Team 2011).

Results

Intraspecific competition

The number of oviposition scars found on pupae of *D. suzukii* increased exponentially as the density of couples of parasitoids also increased F = 1171.71, df = 3, p = 0.0009, Fig 1a). The duration of the biological life cycle of the progeny did not adjust to any regression model (polynomial or exponential), however, it was possible to observe a higher duration when pupae were exposed to more couples of parasitoids (Fig 1b). Not surprisingly, the numbers of attacked pupae increased when they were exposed to a higher number of parasitoids (F = 24.99, df = 3, p = 0.0378, Fig 1c), however, viability was not affected (Fig 1d). Sex ratio of offspring data also did not adjust to any regression model, and was not affected by parasitoid density (Fig 1e), however, in all treatments tested the progeny was female-biased.

Host deprivation

Host deprivation did not affect the duration of biological cycle (H = 2.29, df = 3, p = 0.5145, Table 1). When hosts were offered in alternated days (T3), females parasitized a lower number of pupae when compared to other treatments, meanwhile host-deprived females for three (T4) or seven days (T5) were able to parasitize an amount of pupae comparable to those who were host-provided (T1) (F = 24.57, df = 3, p < 0.0001, Table 1). And as soon as hosts became available, deprived females were able to immediately use their eggs (Fig 2). The different periods of absence of host also affected the total number of offspring generated per female (H = 17.10, df = 3, p = 0.0007, Table 1), and the highest number of offspring was obtained when females were not submitted to any host deprivation (T1) and when they were submitted to 7 days of host deprivation (T5) (146.40 ± 25.55 and 131.47 ± 15.02 parasitoids,

respectively). In addition, sex ratio of offspring was also affected by the treatments ($F = 4.49$, $df = 3$, $p = 0.0386$, Table 1). Ultimately, longevity of males was not affected by host deprivation ($H = 1.90$, $df = 4$, $p = 0.7540$, Fig 3a), whereas longevity of females was affected ($H = 48.50$, $df = 4$, $p < 0.0001$, Fig 3b), and females that never received hosts lived longer (73.33 days).

Discussion

In our study, we verified an increase of oviposition scars when pupae were exposed to a higher density of females. Besides oviposition, these scars may be the result of a behavior of host-feeding or host-evaluation. In the case of parasitoids from the Genus *Trichopria*, adults do not show host-feeding behavior (Carton *et al* 1986), so the scars left on the host may be the result of the insertion of ovipositor for internal evaluation followed by the decision to lay an egg or not. Once female parasitoids find a host, they may insert their ovipositor into their host to evaluate quality of the host or to destroy competing eggs or larvae from other parasitoids before laying their own eggs (Abram *et al* 2019). Most species of parasitoids are able to recognize and reject hosts that were previously parasitized by conspecifics or by themselves (Montoya *et al* 2012). Although parasitized hosts are considered of lower quality, females can lay their eggs on parasitized hosts and this is called superparasitism (Van Alphen 1990). Some of the conditions that lead to the occurrence of superparasitism are the low cost in laying extra eggs, high quality of the host, an increase in the probability of offspring survival due to suppression of the host's immune system and intraspecific competition (Van Alphen 1990, Montoya *et al* 2012). Although we did not dissect pupae to confirm the presence of supernumerary eggs, superparasitism in the Genus *Trichopria* was confirmed by Boulétreau (unpublished data) (see Carton *et al* 1986) and also by O'neil (1973). Thus, the increase of oviposition scars found on pupae exposed to a higher density of females could be due the

occurrence of superparasitism in this species, despite only one adult emerge per parasitized pupae.

The egg-to-adult period was higher in treatments of higher density, probably due to occurrence of superparasitism in the host. The extension of development period of parasitoids in conditions of superparasitism was previously reported for other species of parasitoids, probably due competition for resources (Parra *et al* 1988, Tunca & Kilincer 2009). Host deprivation of parasitoids could lead to self-superparasitism once host became available, resulting in an extension of the development period, as observed when host was exposed to high densities of foundress. However, host deprivation did not result in any change in the duration of the biological cycle, and although self-superparasitism cannot be completely discarded, it seems not so likely to have occurred.

In different competitive environment, the highest percentage of attacked pupae was achieved when pupae were offered to the highest number of foundress females. Although this result was expected, it is interesting that not all the pupae were attacked even in the higher density tested. Patch underexploitation was already observed in other parasitoids (DaSilva *et al* 2016, Tena *et al* 2017). Pierre *et al* (2003) suggested that the decision of leaving unparasitized host in a patch could be due a sampling performed by female parasitoids, and the decision of giving up a patch is taken after a female parasitoid encounters too many parasitized hosts during the exploitation.

Furthermore, the viability of parasitized pupae (i.e. number of emerged parasitoids divided by the number of pupae containing scars) was not affected by density, contrasting with others studies, where competition for host resulted in a higher mortality of parasitoids during their development (Gonzalez *et al* 2007, Cruz *et al* 2018).

The reproductive success of a parasitoid also depends on the ability of females in adjusting their egg production according to host availability (Hougardy *et al* 2005, Carneiro *et*

al 2009). When females are host-deprived, some species can resorpt their oocytes, as a nutritional source, resulting in a decrease in parasitism (Hougardy *et al* 2005), while others can retain their eggs and keep their parasitism rate (Carneiro *et al* 2009). We observed that even after a period of seven days of host deprivation, parasitism was comparable to females which received a daily supply of hosts, thus, oocytes resorption did not seem to occur. However, provision of hosts in alternated days decreased the total number of parasitized pupae per female. Although parasitoids were provided honey during their entire lifetime in all treatments, it is possible that sugar availability did not prevent oocyte resorption. In fact, storage capacity can be insufficient and, when hosts are scarce, ovisorption occurs so the ultimate output of ripe eggs is not affected (Flanders 1950).

Although host availability can be easily manipulated in laboratory conditions to increase reproductive potential of parasitoids that will be released in the field (Hougardy *et al* 2005), the response to host deprivation is species specific. While in some species, provision of hosts to freshly emerged females can stimulate oogenesis, for others it can decrease the remaining reproductive capacity (Fleury & Bouletreau 1993, Hougardy *et al* 2005, Carneiro *et al* 2009). Our results show that host deprivation for a period up to seven days post-emergence do not impair the parasitism capacity of females. Thus, it can be used as a pre-release strategy in cases where release at that particular moment is not possible.

Regarding sex ratio, it is expected that insect population has equal investments in both sexes, resulting in a sex ratio close to 0.5 (Fisher 1930). However, deviation in this proportion are not rare, and can be attributed to ecological, physiological and behavioral factors (Flanders 1939, Peruquetti & Lama 2003). In a study with *T. drosophilae*, a sex ratio close to 0.5 was verified when a higher number of foundress were competing for *D. melanogaster* pupae (Li *et al* 2018), while in our study the proportion of females in offspring was always higher than 0.5, regardless the density. In fact, despite the variation, sex ratio of offspring produced by females

in different competition environments were female-biased. Female-biased offspring is an asset, since females are responsible for both population growth and pest mortality (Montoya *et al* 2012). Nevertheless, when evaluating mean sex ratio of total offspring produced by females during their lifetime (i.e. in the host deprivation bioassay), we found an overall lower percentage of females produced. In most Hymenoptera species, parasitoid sex ratio decrease with maternal age, since after the oviposition of fertilized eggs (which generate females), parasitoids can continue to lay unfertilized eggs (males). This switch occurs probably due to sperm depletion or depletion of spermathecal gland secretions (Kopelman & Chabora 1986, Rossi Stacconi *et al* 2017). It is true that in our study, females were coupled with males during their lifetime, thus, they had plenty opportunity for mating, however, there is no information in literature if *T. anastrephae* performs multiple mating, or if they are able to mate when they are older, and then, replenish their sperm storage. Moreover, the higher percentage of males in progeny was obtained when we offered hosts daily to *T. anastrephae*. Curiously, in Nasonia vitripennis Walker (Hymenoptera: Pteromalidae), host deprivation increased the proportion of males in progeny (King 1962). However, Flanders (1939) suggested that as eggs remain longer in the ovariole of host-deprived females, they have a better chance of being fertilized, and thus produce females.

A trade-off between reproduction and survival is common in insects (Ellers *et al* 2000, Hougardy *et al* 2005). The lack of host deprivation effects on male longevity was expected, since males do not interact with hosts as much as females. Meanwhile, longevity of females varied greatly according to host availability. Completely host-deprived females lived longer than the females submitted to any other treatment. A similar trend was observed in *T. drosophilae*, where host-provided females lived shorter than host deprived ones (Rossi Stacconi *et al* 2017). It has been suggested that the longer life of host-deprived parasitoids is related to egg-resorption, since ovariole has a limited storage capability the non-use of eggs due to host

deprivation leads to the resorption of mature eggs and recycling of nutrients (Jervis et al 2001). However, the longer longevity can also be related to resource reallocation, since host-deprived parasitoids will invest less into egg production and oviposition, and they will invest more into lifespan, in order to enable them to reach more resourceful places (i.e. with plenty of hosts) (Wajnberg *et al* 2012, Bezerra da Silva *et al* 2019).

Here we show that the extrinsic intraspecific competition among females of *T. anastrephae* result in a higher number of oviposition scars on host and an extension of egg-to-adult period, suggesting the occurrence of superparasitism. However, this competitive environment has no detrimental effect on the emergence of the offspring. Meanwhile, females can be host-deprived for a period up to seven days without a negative effect on their capacity to parasitize pupae, however, provision of hosts in alternated days decreased the total number of parasitized pupae per female. Determining the parasitism behavior of a parasitoid in an environment of competition or host deprivation facilitates the understanding of the interaction between parasitoid and host. This knowledge can be applied through the manipulation of this interaction to optimize mass rearing and to provide basic information to improve biological control programs.

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Author Contribution Statement

APK, TS, JGAV, DB, DEN and FRMG planned and designed the research. APK, TS, JGAV and MCM conducted experiments. APK and TS conducted statistical analysis. APK wrote the manuscript. DB, DEN and FRMG contributed material. DEN and FRMG secured funding. All authors read and approved the manuscript.

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Table 1: Average (± SD) duration of biological cycle (days), total number of parasitized pupae per female, total number of offspring generated per female and sex ratio of *Trichopria anastrephae* obtained from *Drosophila suzukii* pupae, when parasitoids were host-deprived for different periods

Treatment*	Egg-adult period (days)^{ns}	Number of parasitized pupae$^{\beta}$	Number of offspring$^{\alpha}$	Sex ratio$^{\beta}$
T1	18.77 ± 0.36	205.40 ± 32.66 a	146.40 ± 25.55 a	0.34 ± 0.20 b
T3	19.33 ± 2.03	119.73 ± 47.70 b	99.07 ± 38.47 c	0.54 ± 0.25 a
T4	18.96 ± 0.29	187.87 ± 51.58 a	123.13 ± 27.17 bc	0.54 ± 0.19 a
T5	18.94 ± 0.26	195.07 ± 31.63 a	131.47 ± 15.02 ab	0.49 ± 0.21 ab

*T1- hosts provided daily; T3- provision of hosts in alternated days; T4- host deprivation for three days; and T5- host deprivation for seven days. T2 (complete host deprivation) is not shown in the table, since parasitoids had no chance to parasitize.
$^{\alpha}$ Means followed by the same letter in the column did not differ by the Dunn test ($p \leq 0.05$).
$^{\beta}$ Means followed by the same letter in the column did not differ by the Duncan test ($p \leq 0.05$).
^{ns} not significant according to the Kruskal-Wallis test ($p \leq 0.05$)

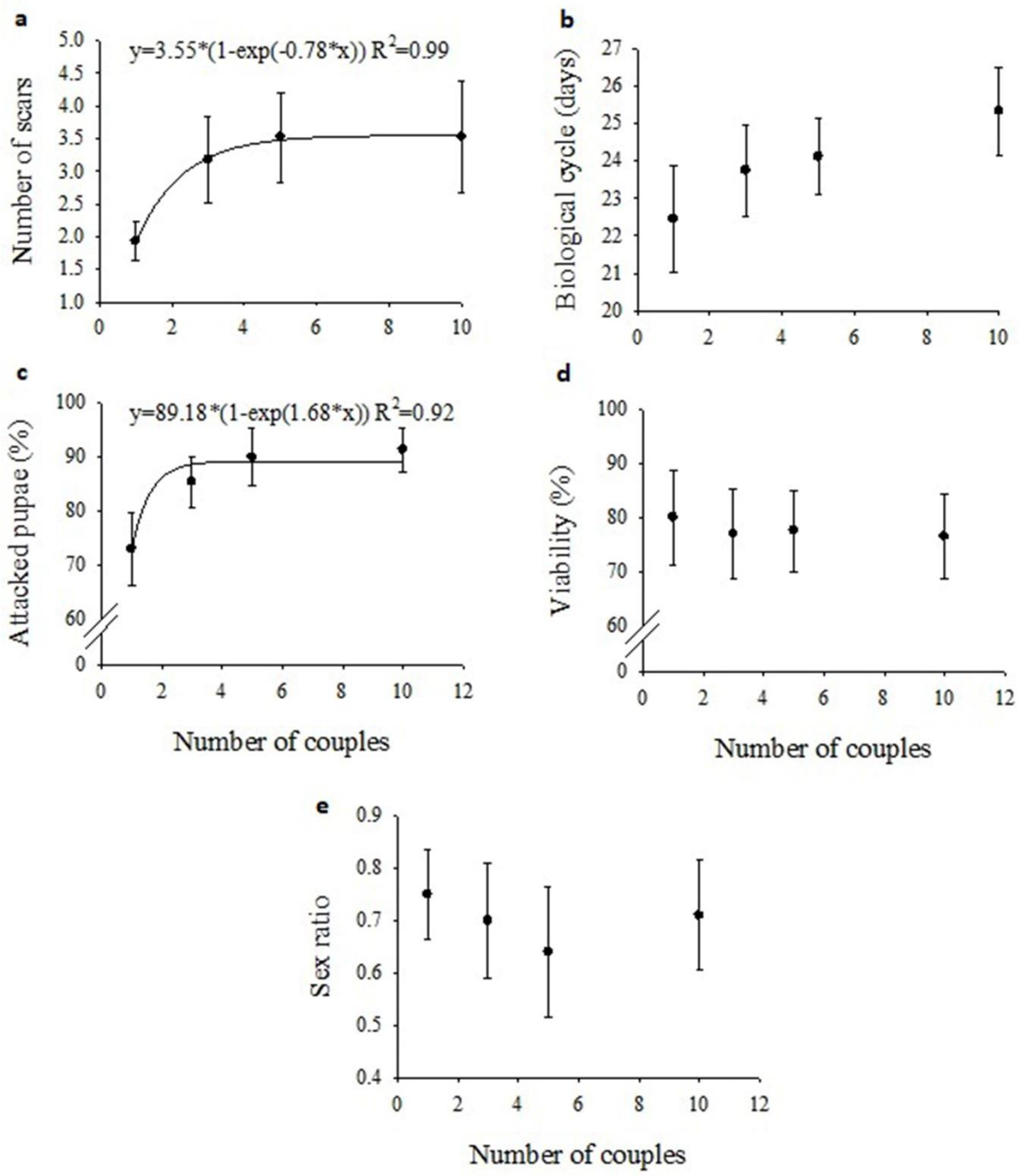

Fig 1: Average number of ovipositor scars (a), duration of egg-adult period (days) (b), attacked pupae (%) (c), viability of parasitized pupae (%) (d), and sex ratio (e) of different densities of *Trichopria anastrephae* reared on *Drosophila suzukii* pupae. Vertical bars represent SD.

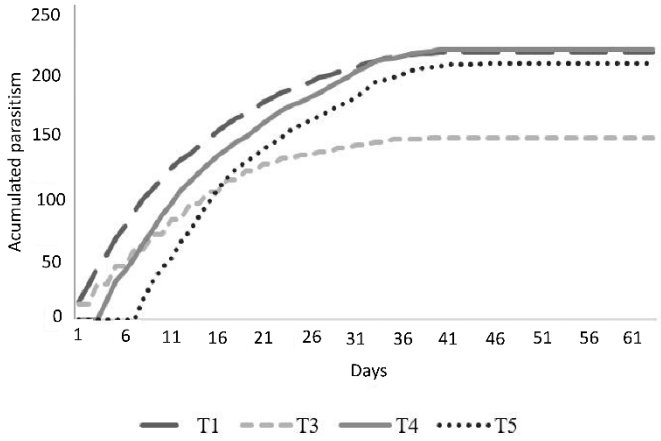

Fig 2: Cumulative number of parasitized pupae of *Drosophila suzukii* by *Trichopria anastrephae* exposed to different periods of host deprivation (T1- hosts provided daily; T2- complete host deprivation; T3- provision of hosts in alternated days; T4- host deprivation for three days; and T5- host deprivation for seven days)

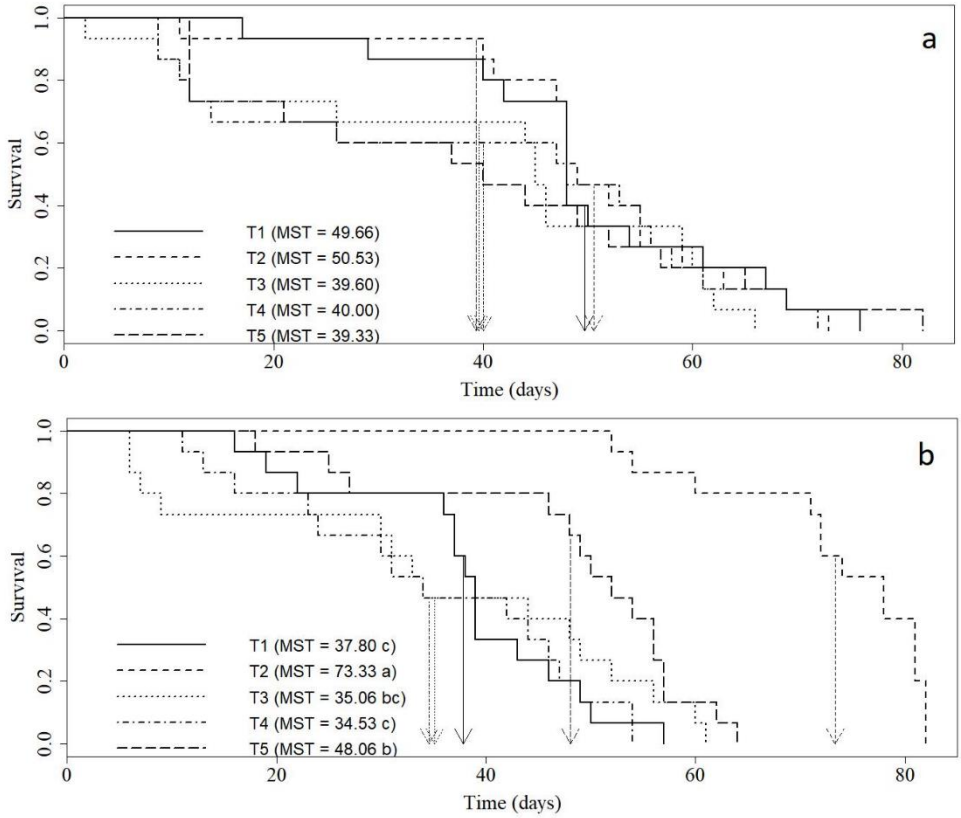

Fig 3: Longevity (days) of males (a) and females (b) of *Trichopria anastrephae* exposed to different periods of host deprivation (T1- hosts provided daily; T2- complete host deprivation; T3- provision of hosts in alternated days; T4- host deprivation for three days; and T5- host deprivation for seven days). The arrows indicate the mean survival time (MST).

Artigo 4 – Biological Control

5- Artigo 4 - *Trichopria anastrephae* as a biological control agent of *Drosophila suzukii* in strawberries

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Highlights

- *T. anastrephae* female is able to recognize strawberry infested with larvae and pupae
- *T. anastrephae* female respond to overripe fruits
- *T. anastrephae* female is able to differentiate infested fruits from uninfested.
- *T. anastrephae* is able to lower de population of *D. suzukii* in strawberries cultivated in greenhouse

Abstract

In South America, the native pupal parasitoid, *Trichopria anastrephae* Costa Lima (Hymenoptera: Diapriidae) is a potential biological control agent of the pest *Drosophila suzukii* Matsumura (Diptera: Drosophilidae). In the present study we 1) examined the behavior of *T. anastrephae* towards different host (*D. suzukii*) and host-substrate (strawberry) cues in choice and non-choice bioassays in laboratory, and 2) examined the density-dependent parasitism of *T. anastrephae* in *D. suzukii*-infested strawberries in a greenhouse. For the behavioral assays we tested infested and non-infested strawberries in a four-chamber olfactometer. For the greenhouse bioassay, we set five walk-in cages with strawberry plants in a greenhouse, released *D. suzukii* adults, and then released different numbers of parasitoids (0, 60, 120, 240 and 360) in each cage. In non-choice bioassays, female parasitoids showed a preference towards *D. suzukii*-infested strawberries containing larvae, pupae or inoculated pupae, or healthy overripe fruits. When given a choice, female parasitoids walked longer over chambers with fruits infested with eggs, larvae or pupae of *D. suzukii*, when compared to healthy uninfested strawberries, and over overripe fruits when compared to unripe or ripe fruits. In the greenhouse assay, we observed an increase in parasitism and a decrease in the number of *D. suzukii* emerging per fruit with an increase in the number of parasitoids released. Our results allow a better understanding regarding the behavior and parasitism of *T. anastrephae* in *D. suzukii*-infested strawberries and provide useful data for potential biological control programs using this parasitoid.

Keywords: pupal parasitoid, spotted-wing Drosophila, olfactometer, behavior, parasitoid release

1. Introduction

The spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is an invasive pest species, native to Southeast Asia, that in the last years expanded its range and, now, is spread in Europe, North and South America and Africa (Bolda et al., 2010; Calabria et al., 2012; Cini et al., 2012; Deprá et al., 2014; dos Santos et al., 2017; Boughdad et al., 2021). After its range expansion, *D. suzukii* rapidly became a major pest in small fruit production, causing an annual economic impact estimated in hundreds of millions of USD, due to yield losses and increased production cost (Goodhue et al., 2011; Knapp et al., 2021). The potential to cause high damage is mainly due to the female's sclerotized and serrated ovipositor that enables them to lay eggs in ripe and ripening healthy fruits (Lee et al., 2011), short life cycle and high fecundity (Emiljanowicz et al., 2014; Tochen et al., 2014) and its large host range (Kenis et al., 2016; Lee et al., 2015; Poyet et al., 2015), that creates a permanent pest pressure.

The main strategy employed for *D. suzukii* management is the use of insecticides, such as spinosyns and pyrethroids (Beers et al., 2011; Bruck et al., 2011; Gress and Zalom, 2019; Van Timmeren et al., 2019). To ensure an effective protection, it is necessary several applications, depending on the susceptibility of the crop, the pest pressure and environmental suitability (Asplen et al., 2015; Shawer et al., 2018). However, the dependency exclusively on insecticides presents potential backlashes, such as insecticide resistance and impacts on non-target species (Desneux et al., 2007; Gress and Zalom, 2019). Also, as *D. suzukii* attacks fruits shortly before harvest, insecticide use is limited due to pre-harvest intervals and maximum residue limits (Diepenbrock et al.,2016). Consequently, research effort has been focused on finding alternative approaches, including semiochemicals (Cloonan et al., 2018), cultural methods (Rendon and Walton, 2019), sterile insects (Krüger et al., 2018), gene drives (Buchman et al., 2018), and biological control (Schetelig et al., 2018; Lee et al., 2019).

Biological control options, such as predators, parasitoids, nematodes, bacteria, fungi and viruses, were studied and demonstrated potential to be used in Integrated Pest Management programs against *D. suzukii* (Garcia et al., 2017; Schetelig et al., 2018; Lee et al., 2019). Biological control agents are mobile and could reduce *D. suzukii* populations in both crop and non-crop areas promoting a sustained suppression throughout the seasons (Lee et al., 2019). Although resident parasitoids associated to *D. suzukii* were found in almost all continents where the pest is present (Rossi Stacconi et al., 2013; Miller et al., 2015; Wollmann et al., 2016, Knoll et al., 2017; Matsuura et al., 2018), few species are able to overcome *D. suzukii* immune response. Due to its high hemocyte load, *D. suzukii* is able to encapsulate eggs from different species of larval parasitoids (Kacsoh and Schlenke, 2012). Thus, the most promising parasitoids that can successfully reproduce on *D. suzukii* are pupal parasitoids.

To date, only a few species of parasitoids have been found associated with *D. suzukii* in South America (Andreazza et al., 2017; De la Vega et al., 2021; Wollmann et al., 2016), including *Trichopria anastrephae* Costa Lima (Hymenoptera: Diapriidae), a resident pupal parasitoid (Cruz et al., 2011) and one most important to biological control of fruit flies in the Neotropical Region (Garcia et al., 2022). Since its association with *D. suzukii*, several aspects of *T. anastrephae* parasitism were studied, including parasitism ability in different laboratory conditions (Krüger et al., 2019; Vieira et al., 2019; 2020), competition with other parasitoid (Oliveira et al., 2021) and toxicological effects of insecticides and essential oils (Bernardi et al., 2017; Geisler et al., 2019; Schlesener et al., 2019; Souza et al., 2020). Results from all these studies demonstrated the potential of *T. anastrephae* as a biological control agent of *D. suzukii* and encourage further studies.

Although most studies were performed using naked pupae only, a recent study showed that *T. anastrephae* is able to discriminate among *D. suzukii* infested and non-infested

blueberry, indicating that this parasitoid can use chemical cues from the interaction among host (*D. suzukii*) and host substrate (fruit) to locate the pest (De La Vega et al., 2021). Similarly, *Trichopria drosophilae* Perkins (Hymenoptera: Diapriidae), a pupal parasitoid found parasitizing *D. suzukii* in North and Central America and Europe, is also able to discriminate among non-crop fruits infested and non-infested by *D. suzukii* (Wolf et al., 2020). Furthermore, semi-field and field experiments showed that released *T. drosophilae* is able to locate and parasitize *D. suzukii* in blueberry, cherry and raspberry (Gonzaléz-Cabrera et al., 2019; Rossi Stacconi et al., 2018; 2019). This parasitoid was also tested in strawberries in field and in greenhouse. After *T. drosophilae* releases in a strawberry field in Italy, only one individual emerged from traps during the trial (Rossi Stacconi et al., 2018), but the reason for this particularly poor performance is not clear. On the other hand, *T. drosophilae* was able to successfully parasitize *D. suzukii* in strawberries in a greenhouse environment, although the low number of parasitoid released was not sufficient to significantly impact the elevated *D. suzukii* infestation (Trottin et al., 2015).

In Brazil, *D. suzukii* imposes a great risk for strawberry production, and heavy economic losses were already reported (Andreazza et al., 2016; Santos 2014), and *T. anastrephae* could be an alternative to control this pest in strawberry orchards. However, it is necessary to evaluate the performance of this parasitoid in *D. suzukii*-infested strawberries. Thus, aiming to evaluate the ability of *T. anastrephae* to parasitize *D. suzukii* in strawberries, we conducted two experiments. In the first experiment, we examined the behavior of this parasitoid towards several host (*D. suzukii*) and host-substrate (strawberry) cues, to identify if *T. anastrephae* is able to discriminate between infested and non-infested strawberries. In the second experiment, we examined the density-dependent parasitism of *T. anastrephae* in *D. suzukii*-infested strawberries in a confined environment (greenhouse).

2. Material and Methods

2.1 Insects rearing

T. anastrephae and *D. suzukii* rearing were conducted under controlled conditions (23 ± 2 °C, 70 ± 10 % r.h. and 12 h of light) at the Laboratory of Entomology, Embrapa Clima Temperado, Brazil. As described elsewhere (Vieira et al., 2019), colonies were initiated from flies and parasitoids collected in nearby organic farms, with new material periodically introduced to maintain colony vigor. Flies were reared on a cornmeal-based diet (Schlesener et al., 2018), and adult flies were held in plastic cages (262 × 177 × 147 mm) (length × height × width), with two side openings (80 × 100 mm), and an opening in the lid (155 × 50 mm), covered with voile cloth. Parasitoids were reared on *D. suzukii* pupae, and adults were held in plastic cages, as the ones described for *D. suzukii*, and fed pure honey (*ad libitum)*.

2.2 Behavioral assays

To test the response of *T. anastrephae* females towards different host and host substrate cues, we conducted behavioral assays in a circular four-chamber olfactometer [adapted from Steidle and Schöller (1997), and previously used for *T. drosophilae* by Wolf et al., (2020)]. The olfactometer used had 15 cm diameter and 6 cm height, and was divided into four equal-sized chambers. One strawberry (~10 g) was placed in one (no choice) or two opposite (choice) chambers of the olfactometer. A plastic mesh was used to cover the olfactometer and a plate (20 cm diameter and 1 cm height) was placed above the mesh. The plate had one hole in the center, where an open 1.5 mL tube containing one parasitoid female (3 to 5 days old, naïve and mated) was inserted. The parasitoid was placed in the tubes 30-60 min before the assay. After leaving the tube, the parasitoid was able to walk freely in the plate, above the chambers, without contacting the fruits. During the tests, the olfactometer was kept in a white plastic basin, and placed under a light source, for evenly distributed light.

For no choice and choice bioassays strawberry fruits containing different potential cues were analyzed (Table 1). All fruits were obtained from plants (cv. San Andreas) cultivated without any pesticide spray, and showed no signs of insect infestation or fungal contamination before they were treated. For treatments containing *D. suzukii* cues, fruits were collected and individually placed in plastic containers (200 mL) with two couples of *D. suzukii*. After 24 hours, *D. suzukii* adults were removed. This treatment induced an infestation of 6-10 eggs per fruit. Infested strawberries were kept for 1 (fruits with eggs), 5 (fruits with larvae) or 8 days (fruits with pupae), before they were used in the bioassays. Non-infested fruits for choice bioassays were collected in the same day, but they were not exposed to *D. suzukii* adults. Artificially damaged fruits were collected and perforated ten times with a '00' entomological pin, which was pierced through the skin only about 1 mm. Fruits with inoculated pupae were kept in the lab for 8 days, then perforated and inoculated with ten 24-h-old *D. suzukii* pupae. Strawberries in different ripening stages were also tested. Unripe (1/3 of the fruit was pink) and ripe (completely red fruit) strawberries were collected in the day before being used for bioassays, while for overripe strawberries, fruits were marked in the plant when they were ripe (completely red), but only collected 7-9 days later, when showing signs of overripening (losing their firmness).

Each parasitoid female was observed for 300 s, and the observation began when the female left the tube. If a female did not leave the tube for 50 s, or moved for less than 150 s during observation period, it was considered non responsive and removed from analysis. The period that each female walked over each chamber was logged using the open-source software Boris (Behavioral Observation Research Interactive Software) (Friard and Gamba 2016). Each female was used only once. After each observation, the olfactometer was rotated 90° and the plate over the olfactometer was exchanged. Each fruit and olfactometer was used three times, and then, fruits were removed and olfactometers were cleaned (with distilled

water and neutral soap, rinsed with 70% ethanol, and dried overnight). For each treatment, in both choice and no choice bioassays, between 20 and 37 responsive females were observed. All bioassays were conducted at 23 ± 2 °C, 70 ± 10% RH, and between 09:00 and 14:00 h.

2.3 Greenhouse assay

We tested the parasitism of different densities of *T. anastrephae* released in walk-in cages (360 × 220 × 200 cm) (length × height × width), placed inside a greenhouse. Inside each walk-in cage we placed 18 potted strawberry plants (cv. San Andreas), each plant containing at least 3 ripe fruits. An artificially infestation of *D. suzukii* (60 couples, 3-5 days old) was performed in each cage. Since *T. anastrephae* is a pupal parasitoid, they were released inside each cage 5 days later than *D. suzukii* infestation, to allow host development. Parasitoid were released in each cage according to treatments (T1- no release (control); T2- 60 parasitoids; T3- 120 parasitoids; T4- 240 parasitoids; and T5- 360 parasitoids), in the same sex ratio as the maintenance rearing (0.7).

To assess fruit infestation and parasitism, strawberry fruits were collected 5, 10 and 15 days after parasitoid release. In each fruit sampling, 20 fruits were randomly collected and individually placed in plastic containers (250 mL), containing a fine layer (~2cm) of vermiculite). Number of flies and parasitoids from each fruit was recorded 30 days after fruit sampling. The experiment was replicate 4 times between October 2019 and February 2021. No pesticide was sprayed during experiments.

2.4 Statistical analysis

For data collected in behavioral experiments, the time that female wasps spent walking over chambers containing fruit vs no fruit, in no choice bioassays, or over the two different treatments, in choice bioassays, was analyzed with Wilcoxon signed rank tests ($p \leq 0.05$). And for data collected in the greenhouse trials, we used a generalized linear model (GLM), with quasipoisson error distribution, to assess the effects of parasitoid release density and time

after parasitoid release on number of *D. suzukii* or *T. anastrephae* per fruit ($p \leq 0.05$). In case of significance, a Tukey's post hoc test was run to compare treatments ($p \leq 0.05$). All statistical analysis were performed in the software R version 4.0.3 (R Core Team, 2020).

3. Results

3.1 Behavioral assays

Parasitoid females walked significantly longer over olfactometer chambers with infested strawberry fruits containing larvae, pupae or inoculated pupae when compared to empty chambers (Figure 1). Similarly, *T. anastrephae* females also walked longer over chambers containing overripe strawberries than over chambers without a fruit (Figure 1). However, damaged fruits, fruits containing *D. suzukii* eggs, unripe and ripe fruits did not affect the length that female parasitoids walked over each chamber of the olfactometer (Figure 1).

When offered a combination of two fruits containing two different potential cues, parasitoid females walked longer over chambers containing fruits naturally infested with eggs, larvae or pupae of *D. suzukii*, when compared to healthy uninfested fruits (Figure 2). Females also walked longer over fruits naturally infested pupae than inoculated pupae, and over overripe fruits when compared to unripe or ripe fruits (Figure 2).

3.2 Greenhouse assay

Based on the number of insects emerging from strawberry fruits, we did not observe an interactive effect between number of parasitoid released and time after parasitoid release for either number of *D. suzukii* ($\chi2=1735.20$, $p=0.3828$) or *T. anastrephae* ($\chi2=338.97$, $p=0.1592$). However, the number of *D. suzukii* significantly decreased with the increase in the parasitoid release density ($\chi2=1842.50$, $p<0.0001$; Figure 3). Comparing to control (no parasitoid release), the number of *D. suzukii* adults emerging per fruit decreased 21.12, 48.20,

59.48 and 66.38% when 60, 120, 240 or 360 parasitoids were released, respectively. On the other hand, the number of *T. anastrephae* adults emerging per fruit increased with the increase in the release density ($\chi 2=374.71$, $p<0.0001$; Figure 3). As expected, no parasitoid emerged from the control, while 0.38 parasitoids/fruit emerged when 360 parasitoids were released.

The number of *D. suzukii* and *T. anastrephae* were affected by the time after parasitoid released ($\chi 2=1776.20$, $p<0.0001$ and $\chi 2=350.44$, $p<0.0001$, respectively; Figure 4). When fruits were sampled 5 days after parasitoid release, we observed the highest numbers of *D. suzukii* (1.91±0.20) and *T. anastrephae* (0.30±0.06) adults emerging from fruits. However, the number of emerging insects started to decrease for both species 10 days after parasitoid release (1.36±0.19 *D. suzukii*/fruit and 0.15±0.03 *T. anastrephae*/fruit), and number of *D. suzukii* emerging adults reached its lowest 15 days after parasitoid release (0.95±0.17), whilst number of *T. anastrephae* were stable after 10 days.

4. Discussion

In the present study, we evaluated the behavior of *T. anastrephae*, a potential biocontrol agent of *D. suzukii* in Latin America, towards *D. suzukii*-infested and non-infested strawberries, as well as the density dependent parasitism and ability to decrease *D. suzukii* population in strawberries cultivated in a greenhouse. Our results indicate that female parasitoids are able to recognize fruits infested with larvae and pupae (naturally and artificially infested) or in overripe stage. Moreover, when given a choice, female parasitoids showed preference towards fruits infested with eggs, larvae and pupae over healthy undamaged fruits, and towards overripe fruits over unripe or ripe fruits. Also, *T. anastrephae* was able to parasitize *D. suzukii*-infested strawberries in greenhouse and lower the pest population, in a density dependent manner.

The ability of a parasitoid to search for a host directly affects its efficacy as a biological control agent, and this behavior is often mediated by compounds emitted by the host substrate (plants), the host and/or an interaction among them (Vet and Dicke, 1992). Our results show that infested fruits with larvae or pupae of *D. suzukii* and overripe fruits elicit a response in female parasitoids, since they preferred to walk over chambers containing these treatments as compared to empty controls. In fact, as a pupal parasitoid, *T. anastrephae* has a better chance of finding a host in fermenting fruits, explaining their preference for fruits in a more advanced decaying stage over empty chambers, even when the fruits were not infested. The use of cues from uninfested fruits by female parasitoids were showed in the tephritid parasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) (Segura et al., 2012; Silva et al., 2007). This larval parasitoid was attracted to fruit cues alone, but the presence of its host larvae in the fruit enhanced the attraction (Silva et al., 2007). Other experiment with this species showed that even used larval medium elicits a positive response in female parasitoids, suggesting that byproducts from larval activity are an important source of chemical cues (Segura et al., 2012).

When given a choice, *T. anastrephae* showed preference for infested strawberries (containing eggs, larvae or pupae) over healthy fruits. Similarly, this species also shows preference for infested blueberries when compared to non-infested fruits (De la Vega et al., 2021). In fact, the authors showed that the development of *D. suzukii* immature stages inside the fruit results in changes in the odor profile of the blueberry, which may result in a higher attraction of *T. anastrephae* to infested fruits. Another species of pupal parasitoid, *T. drosophilae*, is also able to distinguish between *D. suzukii*-infested and non-non infested fruits, in at least five different wild non-crop fruits (Wolf et al., 2020). The larval parasitoid *Asobara japonica* Belokobylskij (Hymenoptera: Braconidae) shows preference for *D. suzukii*-infested cherries, strawberries and blackberries when compared to non-infested fruits (Biondi

et al., 2017). Other larval parasitoids, *Leptopilina japonica* Novkovic & Kimura and *Ganaspis brasiliensis* (Ihering) (Hymenoptera: Figitidae), also respond to fruit volatile cues associated with the presence of *D. suzukii* (Biondi et al., 2021).

We also tested fruits containing inoculated pupae. Fruits for this treatment were picked, brought to the lab, and only inoculated and tested 8 days after, to mimic the time taken by *D. suzukii* to become pupae in naturally infested fruits. In no choice experiments, female parasitoids showed a preference to walk over chambers containing fruits with inoculated pupae compared to empty chambers. But they also spent more time over fruits with naturally infested pupae and overripe fruits (which were harvested in the day of the experiment, but they were harvested 8 days after they were considered ripe). Thus, it was not clear if females were behaving towards fruits with inoculated pupae due to the presence of pupae or the age of the strawberry. In fact, when given a choice between inoculated pupae or healthy fruit (both tested 8 days after picking), female parasitoids did not show any preference, suggesting that the sole presence of host pupae does not seems to be sufficient to elicit a response when compared to old fruits. However, female parasitoids spent more time walking over fruits with naturally infested pupae when compared to fruits with inoculated pupae.

For a biological control agent, the foraging process includes host habitat (or substrate) location, host location, host acceptance and host suitability (Vinson, 1976, 1985). Our results suggest that *T. anastrephae* uses cues from overripe strawberries, that may be indirectly associated with fly infestation, since infested fruit become rotten more rapidly, thus, overripe/rotten fruits are more likely to contain hosts than unripe/ripe fruits. In a longer range, volatile compounds released by overripe fruits may direct parasitoids to habitats with a higher probability of host occurrence (Segura et al., 2012).

Artificially damaged fruits and fruits with eggs did not elicit a response in *T. anastrephae.* These cues are associated to initial phases of infestation, and, as *T. anastrephae* is a pupal parasitoid, they may not be useful in host searching (Hoffmeister and Gienapp, 1999; Segura et al., 2012). On the other hand, infested fruits, containing larvae or pupae of *D. suzukii,* also elicit a positive response in the parasitoid. The pupal stage, used by *T. anastrephae* as host, is motionless and do not feed, resulting in a minimum odor emission (Fischer et al., 2001), and our results indicate that the combination of larvae feeding and pupating in the fruit have an effect on the wasp. Probably, the cues responsible for promoting parasitoid response result from the combination of the host substrate (fruit), the host, and the host-associated microorganisms and/or byproducts (Vet and Dicke, 1992; Hamby and Becher, 2016).

In the greenhouse trial, to guarantee infestation of *D. suzukii*, we released adults of this pest, before releasing parasitoids. The average infestation in our control cage (no parasitoid release) was of 2.32 emerging *D. suzukii* per fruit. This infestation can be considered a low infestation, as found in early season, since in the same region in Brazil, the average number of emerging *D. suzukii* during the growing season ranges between 5 and 10 adults per strawberry (Santos 2014; Wollmann et al., 2020). The management of *D. suzukii* in early season is indicated to reduce or delay pest outbreak (Rossi-Stacconi et al., 2016). Previous studies demonstrated that early augmentative releases of *T. drosophilae* have the potential to suppress the population of *D. suzukii* and reduce pest damage on crops (Rossi-Stacconi et al., 2019). On the other hand, when *T. drosophilae* was tested in a greenhouse with a high infestation of *D. suzukii,* the parasitoid was not effective (Trottin et al., 2015). Here, we showed that *T. anastrephae* is able to reduce *D. suzukii* population depending on the number of parasitoids released, but, ideally, this parasitoid should be also tested also in higher levels of *D. suzukii* infestation.

Despite initial pest population, the number of released parasitoids is another crucial factor for the success of biological control. In our experiments, the different parasitoid release rates resulted in different impacts on *D. suzukii* and *T. anastrephae* population. Although no difference was seen between control and the lowest number of parasitoid released tested, population of *D. suzukii* decreased when 120 parasitoids or more were released. A previous study showed that releasing up to 3000 *T. drosophilae* adults/ha is not sufficient to impact *D. suzukii* population density, however, releasing 4500 parasitoids/ha resulted in a reduction of 50% in *D. suzukii* population in a berry field in Mexico (Gonzales-Cabrera et al., 2019). In our study, releasing 240 and 360 parasitoids per cage resulted in a decrease of approximately 60% in *D. suzukii* population. On the other hand, the number of parasitoids emerging per fruit was low (an average of <1 parasitoid/fruit) even in the highest release ratio. However, it is important to note that this number may be underestimated, since we only considered parasitoids emerging from pupae located in fruits collected from plants. Although there were no fruits on the ground to be collected, *D. suzukii* is able to pupate in the soil (Woltz and Lee, 2017), and such pupae could also be parasitized.

Here, the timing of parasitoid release was planned according to the development of *D. suzukii*, to ensure that pupae would be available for a higher parasitoid success. In nature, it is more difficult to predict the most susceptible period of the host, since are several biotic and abiotic factors involved. However, simulated predictions of pest population were performed for regions in the northern Hemisphere and allow researchers to predict periods when populations are composed by different stages, including pupae (Wiman et al., 2014). This information is useful to obtain the highest efficiency from parasitoid releases and to plan early season management of *D. suzukii* (Pfab et al., 2018; Rossi-Stacconi et al., 2019). Such simulations are not yet available for the southern Hemisphere, but it would be helpful for planning biological control programs.

Besides host availability, another important factor that should be considered before parasitoid release is the temperature range in which the parasitoid is more reproductively active. A recent study found that *T. anastrephae* is able to parasitize *D. suzukii* in temperatures ranging from 10 to 30 °C (Vieira et al., 2020). During our experiments, the average minimum and maximum temperature inside the greenhouse was 20.28 and 31.72 °C (data not presented), respectively, allowing the parasitoid to parasitize hosts. Moreover, in our experiments, we released the parasitoids only once, but multiple parasitoid release, at least in the early stages of fruiting, may be more effective, since increases the tolerance towards suboptimal conditions for the parasitoid (Lopes et al., 2009; Pfab et al., 2018). In nature, where it is not possible to know the exact time of the beginning of infestation as in our experiment, is necessary to find a balance in the season between the beginning of host availability and the pest density, to allow the augmentation of parasitoid density and prevent the increase of the pest population (Pfab et al., 2018).

The results presented in this study provide some initial data that can be useful for biological control strategies using *T. anastrephae.* Our results allow a better understanding of the cues used by *T. anastrephae* in host searching, and their ability to parasitized infested strawberries. This indigenous biocontrol agent, despite being generalist, is an alternative to more specialist parasitoid species identified in *D. suzukii'*s native range (see in Lee et al., 2019), since the introduction of exotic natural enemies may be challenging due to international regulations and risks imposed to resident biodiversity (De la Vega et al., 2021). Moreover, as a local parasitoid, *T. anastrephae* is expected to be well adapted to local environmental conditions, and may not only parasitize *D. suzukii* pupae found in the targeted crop, but also in unmanaged adjacent crops and wild fruits, that may serve as reservoir of *D. suzukii.*

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Author Contribution Statement

APK, DEN and FRMG planned and designed the research. APK, AMG and TS conducted experiments. APK conducted statistical analysis. APK, DEN and FRMG wrote the manuscript. DEN and FRMG contributed material. DEN and FRMG secured funding. All authors read and approved the manuscript.

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Table 1: Cues of *Drosophila suzukii*-infested and non-infested strawberries potentially used by female *Trichopria anastrephae*, tested in a series of no choice and choice bioassays. Rationale for each treatment is also presented.

Treatment	Rationale
Superficially damaged fruit (also tested against healthy fruits, in choice bioassays)	Compounds released by the fruit after being damaged are used as cues by female parasitoids
Fruit with eggs (naturally infested) (also tested against healthy fruits, in choice bioassays)	Compounds derived from egg-laying and adult flies activities are used as cues by female parasitoids
Fruit with larvae (naturally infested) (also tested against healthy fruits, in choice bioassays)	Compounds derived from larval feeding are used as cues by female parasitoids
Fruit with pupae (naturally infested) (also tested against healthy fruits or against fruits with artificially infested pupae, in choice bioassays)	Compounds derived from larval feeding and the presence of host pupae are used as cues by female parasitoids
Fruit with pupae (artificially infested) (also tested against healthy fruits or against fruits with naturally infested pupae, in choice bioassays)	Compounds derived from the presence host pupae are used as cues by female parasitoids
Unripe fruit (also tested against ripe fruits or overripe, in choice bioassays)	Compounds derived from healthy unripe fruits are used as cues by female parasitoids
Ripe fruit (also tested against unripe fruits or overripe, in choice bioassays)	Compounds derived from healthy ripe fruits are used as cues by female parasitoids
Overripe fruit (also tested against unripe fruits or ripe, in choice bioassays)	Compounds derived from healthy overripe fruits are used as cues by female parasitoids

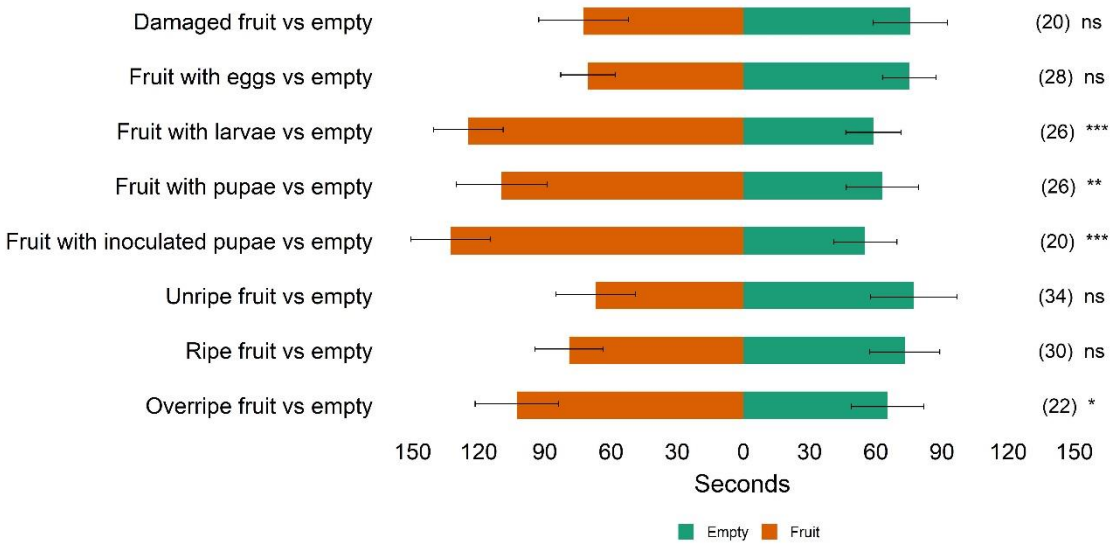

Figure 1. Length of time (seconds) that parasitoid females spent walking over olfactometer chambers containing a fruit with different potential cues vs. no fruit (empty). Values are mean ± SE. Sample size is indicated in the brackets. ns indicates no significant difference, while *, ** and *** indicate a significant difference at 5,1 and 0.1% level, respectively (Wilcoxon signed rank tests)

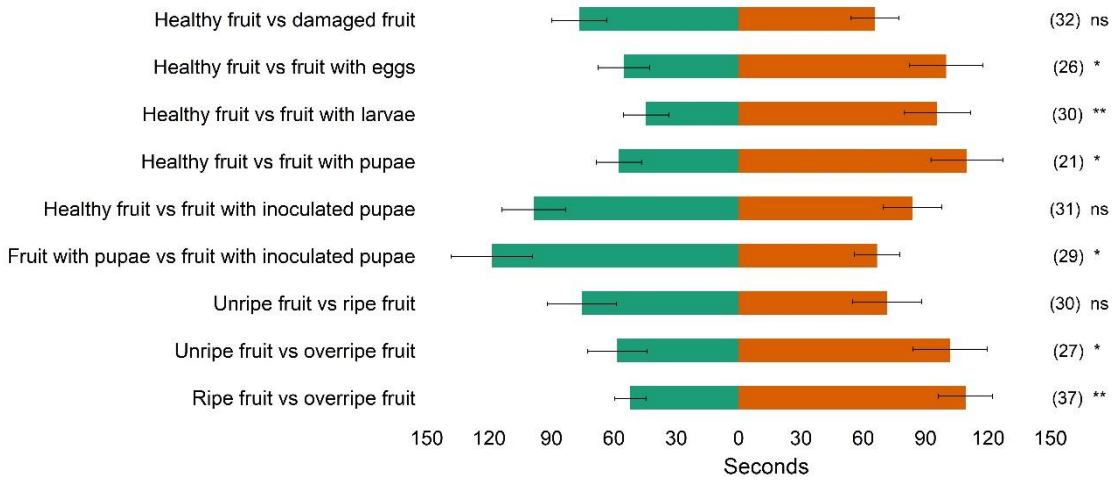

Figure 2. Length of time (seconds) that parasitoid females spent walking over olfactometer chambers containing fruits with different potential cues. Values are mean ± SE. Sample size is indicated in the brackets. ns indicates no significant difference, while *, ** and *** indicate a significant difference at 5,1 and 0.1% level, respectively (Wilcoxon signed rank tests)

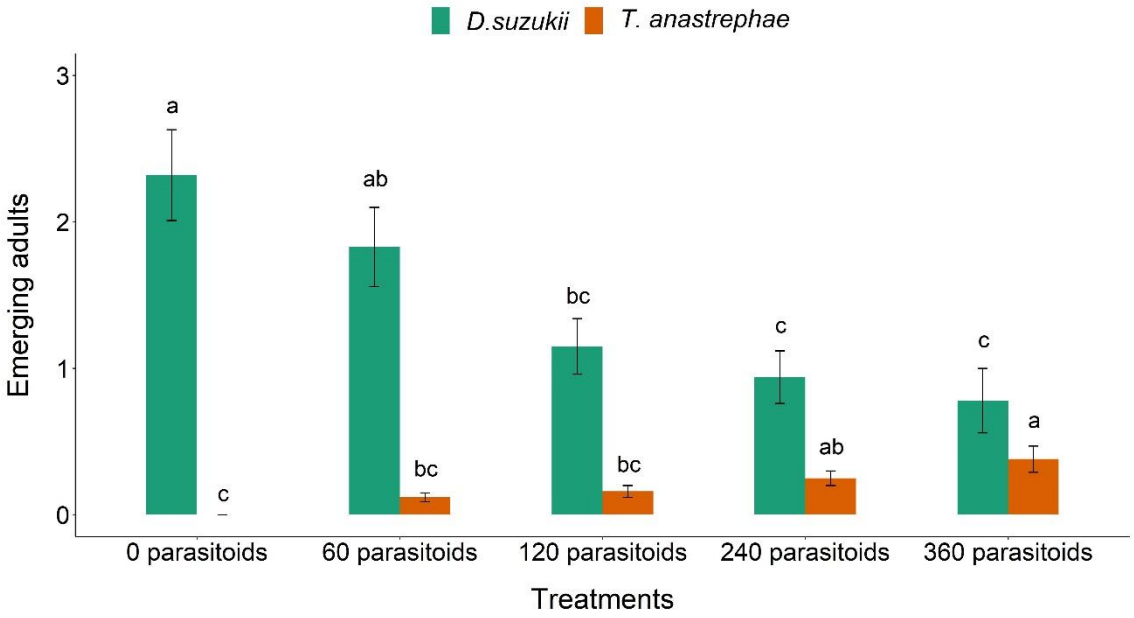

Figure 3. Number of *Drosophila suzukii* and *Trichopria anastrephae* adults emerging per fruit collected from strawberries plants treated with different release densities of *Trichopria anastrephae*.

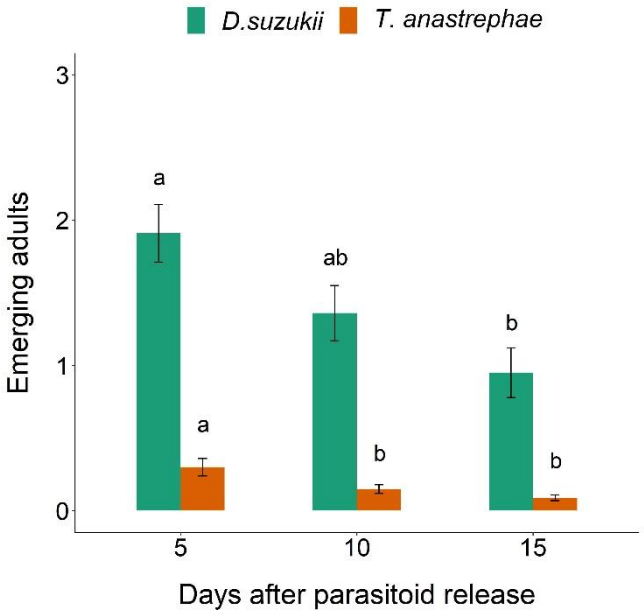

Figure 4. Number of *Drosophila suzukii* and *Trichopria anastrephae* adults emerging per fruit 5, 10 and 15 days after parasitoid release.

6. Conclusões

- As proteínas mais abundantes no veneno de *T. drosophilae* são apolipoforinas, enzimas proteolíticas, endonucleases e toxinas.
- A injeção de veneno de *T. drosophilae* em pupas de *D. melanogaster* afetam a transcrição de genes Turandot, proteases, inibidores de proteases e P450.
- Após a eclosão de *T. drosophilae*, a serosa se dissocia em teratócitos, que crescem com o tempo e expressam principalmente peptídeos antimicrobiais.
- *T. anastrephae* apresentam protandria e a idade dos parasitoides no momento da cópula afeta o número e o sexo da progênie.
- Machos e fêmeas de *T. anastrephae* apresentam poligamia, e o número de cópulas de ambos os sexos afetam a progênie.
- Oferta de hospedeiros em dias alternados afeta negativamente a capacidade de parasitismo de fêmeas de *T. anastrephae*.
- A competição intraespecífica por fêmeas de *T. anastrephae* sugerem a ocorrência de superparasitismo, mas não afetam a viabilidade das pupas parasitadas.
- Fêmeas de *T. anastrephae* são capazes de diferenciar morangos infestados por *D. suzukii.*
- Fêmeas de *T. anastrephae* são capazes de encontrar e parasitar pupas de *D. suzukii* em morangos em casa de vegetação.
- *T. anastrephae* demonstra potencial para ser utilizado no controle biológico de *D. suzukii.*

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