UNIVERSIDADE ESTADUAL DE CAMPINAS

INSTITUTO DE BIOLOGIA

CESAR HENRIQUE MONDINI

CANIBALISMO E *Spiroplasma* EM *Drosophila*

CANNIBALISM AND *Spiroplasma* IN *Drosophila*

CAMPINAS

2022

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Dissertação apresentada ao Instituto de Biologia da Universidade Estadual de Campinas como parte dos requisitos exigidos para a obtenção do Título de Mestre em Genética e Biologia Molecular, na área de Genética Animal e Evolução.

Dissertation presented to the Institute of Biology of the University of Campinas in partial fulfillment of the requirements for the degree of Master of Science in Genetics and Molecular Biology, in the field of Animal Genetics and Evolution.

Orientador: LOUIS BERNARD KLACZKO

ESTE ARQUIVO DIGITAL CORRESPONDE À VERSÃO FINAL DA DISSERTAÇÃO DEFENDIDA PELO ALUNO CESAR HENRIQUE MONDINI E ORIENTADA POR LOUIS BERNARD KLACZKO

CAMPINAS

2022

Ficha catalográfica
Universidade Estadual de Campinas
Biblioteca do Instituto de Biologia
Mara Janaina de Oliveira - CRB 8/6972

M745c

Mondini, Cesar Henrique, 1990-
Canibalismo e *Spiroplasma* em *Drosophila* / Cesar Henrique Mondini. – Campinas, SP : [s.n.], 2022.

Orientador: Louis Bernard Klaczko.
Dissertação (mestrado) – Universidade Estadual de Campinas, Instituto de Biologia.

1. Canibalismo em animais. 2. *Spiroplasma poulsonii*. 3. *Drosophila* - Comportamento. 4. Animais - Alimentos. I. Klaczko, Louis Bernard, 1953-. II. Universidade Estadual de Campinas. Instituto de Biologia. III. Título.

Informações para Biblioteca Digital

Título em outro idioma: Cannibalism and *Spiroplasma* in *Drosophila*
Palavras-chave em inglês:
Cannibalism in animals
Spiroplasma poulsonii
Drosophila - Behavior
Animals - Food
Área de concentração: Genética Animal e Evolução
Titulação: Mestre em Genética e Biologia Molecular
Banca examinadora:
Louis Bernard Klaczko [Orientador]
Andre Luiz Paranhos Perondini
Vera Nisaka Solferini
Data de defesa: 31-05-2022
Programa de Pós-Graduação: Genética e Biologia Molecular

Identificação e informações acadêmicas do(a) aluno(a)
- ORCID do autor: https://orcid.org/0000-0002-4677-3493
- Currículo Lattes do autor: http://lattes.cnpq.br/9098663872703684

Campinas, 2022

COMISSÃO EXAMINADORA

Prof. Dr. Louis Bernard Klaczko

Prof. Dr. Andre Luiz Paranhos Perondini

Profa. Dra. Vera Nisaka Solferini

Os membros da Comissão Examinadora acima assinaram a Ata de Defesa, que se encontra no processo de vida acadêmica do aluno.

A Ata da defesa com as respectivas assinaturas dos membros encontra-se no SIGA/Sistema de Fluxo de Dissertação/Tese e na Secretaria do Programa de Genética e Biologia Molecular do Instituto de Biologia.

DEDICATÓRIA

Dedico esse trabalho à Telma Paschoalino, minha mãe, que sempre me fez ver a educação e o saber como um caminho para a libertação, e que me deu o apoio e o suporte necessários para minha jornada acadêmica.

AGRADECIMENTOS

Agradeço aos membros da banca de qualificação, Vera Solferini, Fabio Papes e Antônio de Carvalho pelos preciosos comentários.

Agradeço aos membros da banca de defesa.

Agradeço aos colegas Claudete, Fabiana, Felipe, Gustavo, Iuri, Klélia, Marcos, Mitsue, Rafael e Thaís pelo auxílio, companhia e companheirismo.

Agradeço aos meus irmãos, Debora e Leandro, por estarem sempre ao meu lado.

Agradeço aos amigos que tornaram esse trajeto mais agradável e menos solitário.

Agradeço ao Marcus, pelo companheirismo e pelo carinho.

Agradeço, finalmente, ao professor Louis Bernard Klaczko que, além de me orientar em minha trajetória científica, foi sempre uma inspiração e um auxílio precioso e certeiro.

O presente trabalho foi realizado com apoio da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Código de Financiamento 001.

RESUMO

Canibalismo e infecção por *Spiroplasma poulsonii* são aspectos recentes e ainda insatisfatoriamente compreendidos em *Drosophila melanogaster*.

O comportamento das infecções de *S. poulsonii* em *Drosophila* em cenários naturais é pouco conhecido. Ao levantar a prevalência na cidade de Campinas, não encontramos uma única mosca infectada, quando utilizamos a maior coleta já feita para estimativas do tipo. Os dados indicam uma potencial extinção, senão a prevalência mais baixa já estimada, e a infecção havia sido detectada na cidade anteriormente. A baixa (senão inexistente) infecção é um dos componentes do complexo cenário das infecções do tipo *male-killer,* como é a infecção de *S. poulsonii* em *Drosophila*, posto que a infecção tende a matar machos para maximizar sua transmissão vertical, mas isso possui um alto custo populacional para seu hospedeiro. Uma das principais soluções para este conflito é a realocação de recursos via canibalismo.

Canibalismo é um comportamento com importantes reflexos na ecologia e na história evolutiva de espécies, e as principais evidências de sua ocorrência em *Drosophila* são de menos de uma década. Neste trabalho, avaliamos a existência de canibalismo de forma mais ampla no gênero *Drosophila*, e procuramos caracterizar em que estágios pode ocorrer, bem como alguns aspectos evolutivos associados. Usando sete espécies do gênero, através da observação de agregação e comportamento alimentar, detectamos a ocorrência de canibalismo, praticado por larvas, para todas as espécies, em presas larvas e presas adultas. Ademais, cinco das sete espécies também canibalizaram ovos. Observando a integridade de ovos, detectamos tempo de exposição à canibalização e densidade de potenciais canibais como fatores diretamente proporcionais ao rompimento da barreira protetiva dos ovos. Também obtivemos evidência do grande potencial adaptativo de canibalismo na primeira ocorrência observada de desenvolvimento ovo-adulto exclusivamente através de canibalismo competitivo. Finalmente, por meio de seleção artificial do tamanho em ambientes onde canibalismo era favorável, obtivemos linhagens que canibalizaram em frequências distintas, mas que não apresentavam tamanho médio diferente, indicando que canibalismo pode ser selecionado indiretamente por tamanho e que o mecanismo principal que modula canibalismo é em algum grau independente de voracidade em geral. Sendo canibalismo ubíquo, seria ele então o responsável

pela realocação de recursos de irmãos mortos pela infecção de *S. poulsonii* em *Drosophila* para as irmãs?

Não há na literatura benefícios incontroversos da infecção de *S. poulsonii* derivados da morte dos machos. Usando de privação nutricional extrema, analisamos os efeitos da infecção em *Drosophila*. Nossos resultados mostraram que larvas filhas de mães infectadas por *S. poulsonii* eram maiores às 72h e chegaram ao estágio adulto mais de um dia antes das filhas de mães não infectadas, quando em *stress* nutricional. Além disso, a infecção aumentou a frequência de canibalismo em indivíduos infectados. Temos, portanto, todos os elementos necessários para a realocação de recursos via canibalismo oferecer benefícios ecológicos para linhagens infectadas em cenários de privação alimentar, e assim apresentar uma solução para o conflito *male-killer*. No entanto, a relação causal entre canibalismo e tamanho/tempo desenvolvimento ainda precisa ser experimentalmente observada para que esta conexão seja estabelecida.

Palavras-chave: *Drosophila*, *Spiroplasma poulsonii*, canibalismo, *male-killer*.

ABSTRACT

Cannibalism and *Spiroplasma poulsonii* infection are recently investigated and insufficiently comprehended aspects in *Drosophila melanogaster*.

The behavior of *S. poulsonii* infections in *Drosophila* in natural scenarios is not well known. Assessing the prevalence in Campinas city, we have not found a single infected fly using the largest sample ever made for similar estimates. Data indicate potential extinction, or the lowest estimate ever obtained, and the infection had previously been found in Campinas. The low (or inexistent) infection is one of the components of the intricate scenario of male-killer infections, as the one of *S. poulsonii,* given that the infection tends to kill males to maximize vertical transmission, but this leads to a high population cost to the host. One of the main solutions for this conundrum is the resource reallocation via cannibalism.

Cannibalism is a behavior with important consequences in the ecology and evolutionary history of species, and the main evidences of its occurrence in *Drosophila* are no older than a decade. In this work, we evaluated the existence of cannibalism in a broader spectrum inside the *Drosophila* genus, and we aimed to characterize in which stages it might take place, as well as other associated evolutionary aspects. Using seven species from the group, through observation of aggregation and feeding behavior, we detected the occurrence of cannibalism, by larvae, in all species, when prey was larvae or adults. Moreover, five out of the seven species also cannibalized eggs. Observing egg integrity, we detected exposure time to cannibals and potential cannibal density to be directly proportional to the rupture of the protective layer of eggs. We also obtained evidence of the important adaptive potential of cannibalism in the first observed instance of egg-adult development exclusively through competitive cannibalism. Finally, by means of artificial selection of size in environments where cannibalism was favorable, we obtained lineages that cannibalize in different rates, but do not differ in average size, which indicates that cannibalism can be indirectly selected through size and that cannibalism's main modulative mechanism is in some degree independent from general voracity. Considering that cannibalism is ubiquitous, would it then be the responsible for reallocating the resources from brothers killed by *S. poulsonii* infection in *Drosophila* to their sisters?

In the literature there are no uncontentious benefits of the *S. poulsonii* infection which are derived from the death of males. Using extreme nutritional

deprivation, we analyzed the infection effects in *Drosophila*. Our results show that larval daughters of infected females were larger at 72h and reached the adult stage more than one day earlier than daughters of uninfected mothers, when in nutritional stress. Furthermore, the infection increased the frequency of cannibalism in infected individuals. We have, therefore, all the elements needed for the reallocation of resources via cannibalism to offer the ecological benefits to infected lineages in food deprivation, and so to present a solution to the male-killer conundrum. However, the cause-effect link between cannibalism and size/development time still needs to be experimentally observed so that this connection can be stablished.

Keywords: *Drosophila*, *Spiroplasma poulsonii*, cannibalism, *male-killer*.

SUMÁRIO

1. INTRODUÇÃO

Em 2000, fortuitamente, nosso grupo de pesquisa encontrou um elemento genético egoísta que matava machos em *Drosophila melanogaster* (Montenegro *et al*., 2000). Posteriormente, em colaboração com o Dr. G. D. D. Hurst tal elemento foi identificado como a bactéria *Spiroplasma poulsonii*, um parasita reprodutivo com efeito *male-killer* (Montenegro *et al*., 2005). Posteriormente, essa infecção foi também encontrada na América do Norte (Watts *et al*., 2009) e na África (Pool *et al*., 2006).

Parasitas reprodutivos são simbiontes herdáveis que manipulam a reprodução de seu hospedeiro de modo a aumentar sua própria transmissão para a próxima geração (Bandi *et al*., 2001). Simbiontes que são transmitidos uniparentalmente pela linhagem materna tendem, por pressão seletiva, a manipular a reprodução aumentando o número de fêmeas ou reduzindo o número de machos do hospedeiro na população (Drew *et al*., 2019). Ao menos um terço das espécies conhecidas de artrópodes é infectado por algum parasita maternalmente transmitido que manipula seus hospedeiros (Duron *et* al., 2008; Jaenike, J., 2015). Entre tais manipuladores reprodutivos estão *Rickettsia sp*., *Flavobacterium sp*., *Cardinium sp*., *Spiroplasma sp*. e *Wolbachia sp.* (Duron *et al*., 2008). *D. melanogaster* (subgênero *Sophophora*), é naturalmente parasitada por apenas dois gêneros de simbiontes herdáveis, *Wolbachia*, numa interação já amplamente estudada, e *Spiroplasma*, cuja infecção, descoberta em nosso grupo de pesquisa, ainda requer atenção (Mateos *et al*., 2006).

Spiroplasma é um gênero de bactérias helicoidais da classe *Mollicutes*, gram-positivas, sem parede celular, com divisão celular longitudinal (Ramond *et al*., 2016), que obrigatoriamente parasita artrópodes, pelo menos em uma fase de seu ciclo de vida (Whitecomb & Tully, 1982; Cacciola *et al.,* 2017; há exemplos em: Ding *et al.,* 2013; Xiu *et al.,* 2015; Hayashi *et al*., 2016). Infectam entre 5 e 10% das espécies de inseto (Duron *et al*., 2008), dentre as quais estão 17 espécies de *Drosophila* (Haselkorn & Jaenike, 2015). Estudos envolvendo *Spiroplasma* têm sido prolíficos, com novas espécies ainda sendo isoladas (Nai *et al*., 2014), observação inédita da formação de biofilmes (Bastian *et al*., 2012), modelagem de motilidade (López & Lauga, 2020), identificação de expansão geográfica (Cockburn *et al*., 2013; Schneider *et al*., 2019), e verificação de infecção sistemática inédita de *Spiroplasma* em humanos (Aquilino *et al*., 2015), entre outros.

Spiroplasma possui duas categorias de infecção: endossimbionte e não-endossimbionte (Paredes *et al*., 2015). *S. poulsonii* pertence à primeira categoria, de forma que é um parasita reprodutivo que coloniza a hemolinfa e é transmitido por transmissão vertical transovariana na maioria dos casos (Montenegro *et al.*, 2005; Anbutsu & Fukatsu, 2006), embora casos de transmissão horizontal tenham ocorrido e tenham sido fundamentais para a distribuição atual do gênero (Haselkorn *et al*., 2009). Como *S. poulsonii* é responsável por *male-killing* em *D. melanogaster* (Montenegro *et al.*, 2000), este é um endossimbionte que, ao induzir a morte dos machos, maximiza sua transmissão vertical, e que, portanto, modifica a história evolutiva do seu hospedeiro para otimizar a sua própria. Dado que a infecção mata os filhos de fêmeas infectadas, surge a questão de até que ponto esse efeito androcida é custoso para o hospedeiro e, portanto, para a própria bactéria infectante.

Do ponto de vista matemático, uma infecção de um agente que causa *male-killing* e que não possua transmissão vertical perfeita (*i.e.* 100% das fêmeas infectadas transmitam sua infecção às filhas), como é o caso de *S. poulsonii* em *D. melanogaster* (Montenegro *et al.,* 2005), inevitavelmente acabaria extinta. Numa análise teórica, uma infecção como esta, de transmissão vertical imperfeita, só pode perseverar ao longo do tempo evolutivo por três mecanismos (Hurst & Majerus, 1993): i) realocação de recursos (direta, com irmãs comendo irmãos, ou indireta, reduzindo a competição); ii) fuga de endocruzamento, dado que irmãos e irmãs não acasalam; iii) facilitação e/ou viabilização de transmissão horizontal. Porém, para todos os mecanismos, é fundamental que o fenômeno seja dependente da morte dos machos. Para exemplificar tal afirmação temos o caso de Xie e colaboradores (2014), que verificaram que *Spiroplasma* que causa *male-killing* em *D. melanogaster* protege a mosca de infecções de vespas parasitóides. No entanto, dado que tal benefício em *fitness* não depende da morte dos machos, ele, isoladamente, não justifica por que tal infecção persiste, mesmo quando envolve complexas interações com o sistema imune do hospedeiro (Alvear *et al.*, 2021). O questionamento que nasce, portanto, é se há alguma vantagem ecológica/evolutiva que a infecção de *S. poulsonii* cause em *Drosophila* e que seja produto intrínseco da morte dos filhos de mães infectadas.

As evidências acerca da hipótese de realocação de recursos de *S.* poulsonii em *D. melanogaster* são ambíguas: enquanto não se encontrou vantagem alguma em fecundidade ou sobrevivência das filhas de fêmeas infectadas em laboratório (Montenegro *et al*., 2006), há indícios que fêmeas infectadas na natureza sejam mais

fecundas (Martins *et al*., 2010). Além disso, foram encontradas evidências de que fêmeas infectadas no laboratório se desenvolvem mais rapidamente, mas a relação causal entre infecção e tempo de desenvolvimento ficou turva devido a fatores de confusão oriundos da densidade larval. Excluindo densidade como uma variável, experimentos seriam capazes de identificar com maior clareza se, e o quanto *S. poulsonii* afeta o tempo de desenvolvimento de filhas de mães infectadas em *D. melanogaster*, numa manifestação da hipótese de realocação de recursos. Considerando ainda que densidade pareceu ser diretamente proporcional ao efeito de realocação de recurso, seria frutífero investigar a relação *S. poulsonii* e *D. melanogaster* em situações de privação extrema de nutrientes, onde realocação de recursos poderia manifestar-se mais claramente e através de sua forma direta, *i.e.* irmãs canibalizando os irmãos mortos.

Mas, para tal, faz-se necessário compreender com clareza se e como canibalismo se manifesta em *D. melanogaster,* e na literatura estas investigações são recentes, restritas e pouco abundantes. Um experimento de 1990, realizado por Gregg e colaboradores, identificou o consumo canibal de carcaças de adultos de algumas espécies de *Drosophila*. Apenas em 2004 outra investigação nesse sentido foi feita no gênero, por Huey e colaboradores, que não encontraram evidências de consumo de carcaças de adultos por outros adultos de *D. melanogaster.* Quase uma década depois foram encontradas evidências que apontavam canibalismo predatório de larvas por larvas mais jovens (Vijendravarma *et al.,* 2013), e posteriormente tais conclusões foram expandidas para consumo intra-específico de ovos e adultos de *D. melanogaster* (Ahmad *et al.,* 2015). Em seguida verificou-se que *D. melanogaster* protege seus ovos de canibalismo via uma camada de feromônios (Narasimha *et al.,* 2019), mas possivelmente tal proteção decaia com o tempo e permita o consumo canibal de ovos por larvas (Khodaei & Long, 2020).

Embora insuficientemente investigado em *Drosophila*, canibalismo é ecologicamente relevante, dado que pode afetar o valor adaptativo ao permitir a obtenção de recursos em situações de estresse alimentar. Isto ocorre em diversos grupos animais, desde insetos (Gomi *et al*., 2015), a peixes (Baras & Jobling, 2002) e até mesmo bactérias (González-Pastor J., 2011). Há evidências para acreditar que em espécies em que há competição entre irmãos, é mais provável que ocorra canibalismo dos indivíduos com menor *fitness* potencial, tanto em relação à viabilidade (Dugas *et al.,* 2015), quanto em relação ao parentesco (Schultner *et al., 2014*). Como

resultado, canibalismo de irmãos como um todo tende a ser adaptativo para a mãe (através de *fitness* inclusivo) quando em privação alimentar (Osawa, N., 1992; Perry & Roitberg, 2005). Tais vantagens são claramente ligadas a agentes androcidas pelos trabalhos de Michaud e Grant (2004), e de Nakamura e colaboradores (2006): trabalhando com coccinelídeos (joaninhas), o primeiro aponta que fêmeas canibais desse grupo são maiores do que as suas conspecíficas não canibais; o segundo indica que devido ao fenótipo *male-killing* causado por *Spiroplasma*, um maior número de ovos não viáveis induz um aumento de 4 a 14 vezes da taxa de canibalismo de ovos; em conjunto, fêmeas infectadas produzem filhas que canibalizam com maior frequência, tornam-se maiores, e isso resulta numa alta taxa de compensação em *fitness* inclusivo para a mãe, tornando-se adaptativo e viabilizando a prevalência de tal infecção ao longo do tempo evolutivo. Há, nas moscas *Drosophila* infectadas com *S. poulsonii,* alguma manifestação análoga, em que canibalismo gera vantagens competitivas em irmãs que canibalizam os irmãos mortos pelo efeito *male-killing* da infecção?

Este projeto pretende, portanto, investigar a existência de canibalismo em diversas espécies do gênero *Drosophila*, bem como em diferentes estágios de desenvolvimento. Verificar sua importância como fonte alimentar em privação alimentar extrema e a relação evolutiva de tamanho e canibalismo. Ato contínuo, objetivamos compreender se a taxa de canibalismo pode ser modulada por infecções de *S. poulsonii* em *D. melanogaster*, e se, em severa privação nutricional, fêmeas infectadas possuem vantagens ecológicas em relação a fêmeas não infectadas. Finalmente, o projeto propõe-se a revisitar a infecção na população natural de Campinas, local da primeira ocorrência descrita da infecção na literatura.

1.1 OBJETIVOS

ARTIGO 1: estimar a prevalência da infecção de *S. poulsonii* em *D. melanogaster* na região metropolitana de Campinas, São Paulo, Brasil.

ARTIGO 2:

a. verificar a ocorrência de canibalismo por larvas em ovos, larvas e adultos de *Drosophila melanogaster, Drosophila simulans, Drosophila ornatifrons, Drosophila bandeirantorum, Drosophila immigrans, Drosophila virilis* e *Drosophila mediopunctata*.

b. verificar se canibalismo pode ser selecionado indiretamente.

c. verificar se canibalismo pode, como fonte exclusiva de nutrição, garantir que indivíduos cheguem ao estágio adulto em um ambiente de competição direta.

ARTIGO 3:

a. verificar se *S. poulsonii* aumenta o canibalismo larval em *D. melanogaster*

b. verificar se, em privação nutricional extrema, a infecção por *S. poulsonii* proporciona vantagens competitivas (tamanho e tempo de desenvolvimento) para as filhas de mães infectadas.

2. ARTIGO 1: Estimativa de prevalência de *S. poulsonii* em Campinas

Artigo publicado pelo Drosophila Information Service.

TITLE*:* *SPIROPLASMA POULSONII* (ENTOMOPLASMATALES, SPIROPLASMATACEAE) PREVALENCE ESTIMATE INDICATES LOCAL EXTINCTION OF INFECTION WITHIN A *DROSOPHILA MELANOGASTER* (DIPTERA, DROSOPHILIDAE) NATURAL POPULATION IN CAMPINAS, SÃO PAULO STATE, BRAZIL.

Authors: Cesar Henrique Mondini, Louis Bernard Klaczko

Institutional Affiliation: Universidade Estadual de Campinas – UNICAMP, Instituto de Biologia, Departamento de Genética, Evolução e Bioagentes, Laboratório de Biodiversidade Genética e Evolução de *Drosophila*, SP, Brazil

Running Title: Local extinction of *S. poulsonii*

Keywords: endosymbiont; male-killing; suicide king.

Corresponding author:
Cesar Henrique Mondini
João Puche st., 116, Sumaré, SP, Brazil, 13172455
cesarmondini@hotmail.com
ORCID ID: 0000-0002-4677-3493

2.1. MAIN TEXT

Spiroplasma poulsonii is an endosymbiont associated with *Drosophila* and it frequently causes the death of the male offspring of infected mothers in early developmental stages (Anbutsu and Fukatsu, 2011; Ventura *et al.*, 2012). *Spiroplasma* is the only endosymbiont that causes the death of males in *Drosophila melanogaster*, and this effect leads to an evolutionary conundrum: how could a costly infection that causes the fitness of males to plummet to near zero to be maintained throughout evolutionary time? Perhaps there is no way that these infections can persist, and would extinguish themselves (*i.e.* Suicide King – Dybdahl and Storfer, 2003).

Although there are theoretical explanations for the maintenance of the prevalence of male-killers (Hurst and Majerus, 1993), and some works present evidence of those explanations for *Spiroplasma* infections in *D. melanogaster*, as horizontal transmission (Jaenike *et al.*, 2007), or induction of defensive phenotypes to pathogens in the hosts (Mateos *et al.*, 2016; Hamilton *et al.*, 2016; Paredes *et al.*, 2016), no evidence that fulfilled all the theoretical criteria was found to date. This lack of information can lead to the conclusion that these infections would disappear in natural populations, and a way to assess that is through the evaluation of the infection status of flies in nature. Such investigations, however, are few, totalizing four papers (Montenegro *et al.*, 2005; Pool *et al.*, 2006; Watts *et al.*, 2009; Ventura *et al.*, 2012). From these, only the works of Montenegro and Ventura evaluated samples of more than 50 females. Due to the poor assessment of these prevalences, we here estimate the frequency of this infection using 251 females collected in the region of Campinas, Brazil.

Flies were captured using bottles with standard cornmeal- molasses medium and funnels placed for 1-14 days inside residences in the District of Barão Geraldo, Campinas, and in Sumaré, both in São Paulo State, Brazil. Thirty-two collections were held from February to December of 2016 (Table 1). Over 500 flies were collected, and after final identification we obtained 251 D. melanogaster females that produced offspring. They were screened using PCR. DNA extractions used 5-10 two-weeks old daughters, and an alcohol-salt method (Aljanabi and Martinez, 1997). PCR reactions were carried out with the primers used by Montenegro *et al.* (2005) for the 16S rDNA gene of Spiroplasma and the primers listed by Simon *et al.* (1994) for

the CoI gene of D. melanogaster, used to assess the quality of the DNA extraction and PCR amplification.

From the 251 isofemales lines tested, none was diagnosed as infected with *Spiroplasma*. Using Wilson's method (Brown *et al.*, 2001) with a confidence level of 95%, the estimated prevalence interval is 0-1.51%. The estimate maximum value (1.51%) is lower than every prevalence obtained before (Table 2). Moreover, the number of captured females used is higher than any other in the literature, indicating that the inexistence of positively diagnosed females is not a sampling or methodological error, but effect of the very low frequency of the infection.

Beyond the evolutionary paradox afore mentioned, *Spiroplasma* transmission is known to be sensitive to high and low temperatures (Anbutsu *et al.*, 2008; Montenegro *et al.*, 2004) as well as the age of the mother (Kageyama *et al.*, 2007), and these factors could be minimizing the frequency of this infection in the region of Campinas. It could also be a direct observation of a temporal reduction that the infection is going through, as a trend for reduction in prevalence was observed before (Ventura *et al.*, 2012). Two strategies to disentangle these questions would be to run another round of prevalence estimation in Campinas in a few years, and other would be to make the same procedure now in the populations whose prevalence had previously been assessed.

2.2. ACKNOWLEDGEMENTS

M.R.D. Batista, M.T. Brianti, L.F.M. Bartoletti, P.J. Bergamo, F. Uno, R.E.S. Penha and G.B. Passos for helping with the collection. K.A. Carvalho and C. Couto for technical support. I.M. Ventura for insightful tips. CAPES, CNPq and FAPESP for financial support.

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2.4. TABLES

Table 1. Collection information of tested isofemale lines

Collection Date (2016)	GPS coordinates	Putative *D. melanogaster**	*D. melanogaster* isofemale lines tested **
Feb 21 – Feb 27	-22°49'05", -47°05'21"	58	13
	-22°54'01", -47°03'16"	45	38
Feb 18 – Feb 23	-22°54'01", -47°03'16"	11	10
Feb 24 – Feb 29	-22°49'38", -47°04'44"	16	10
Feb 28 – Feb 29	-22°49'38", -47°06'13"	4	0
Jul 19 – Jul 28	-22°49'05", -47°05'21"	8	4
Jul 12 – Jul 28	-22°49'02", -47°05'23"	3	3
	-22°49'17", -47°05'38"	3	1
Aug 19 – Aug 16	-22°49'05", -47°05'21"	12	7
	-22°49'02", -47°05'23"	15	8
Aug 15 – Aug 20	-22°49'05", -47°05'21"	3	1
	-22°49'02", -47°05'23"	26	15
Aug 29 – Sep 08	-22°49'02", -47°05'23"	12	9
	-22°49'05", -47°05'21"	11	7
Sep 10 – Sep 15	-22°49'05", -47°05'21"	26	5
	-22°49'02", -47°05'23"	9	4
	-22°49'17", -47°05'38"	20	11
Sep 16 – Sep 23	-22°49'05", -47°05'21"	1	0
	-22°49'02", -47°05'23"	3	2
	-22°49'17", -47°05'38"	0	0
Sep 18 – Sep 22	-22°47'16", -45°05'07"	8	4
Sep 22 – Sep 26	-22°47'16", -45°05'07"	9	2
Sep 27 – Sep 29	-22°49'38", -47°06'13"	11	1
Sep 23 – Sep 29	-22°49'05", -47°05'21"	32	12
Oct 05 – Oct 08	-22°49'05", -47°05'21"	86	24
Oct 28 – Nov 04	-22°49'05", -47°05'21"	3	3
Nov 08 – Nov 15	-22°48'33", -47°16'59"	31	25
Nov 10 – Nov 16	-22°54'38", -47°05'39"	18	3
Nov 16 – Nov 24	-22°49'05", -47°05'21"	18	13
Nov 20 – Nov 24	-22°54'38", -47°05'39"	41	2
Nov 22 – Nov 28	-22°48'33", -47°16'59"	15	0
Dec 09 – Dec 11	-22°49'38", -47°06'13"	25	14
TOTAL		**583**	**251**

*putative *D. melanogaster* females were then confirmed by the analysis of the male genitalia.

**diagnostic was performed through PCR reactions using 5 to 10 daughters from each female.

Table 2. Estimates of *Spiroplasma* infection within *D. melanogaster* natural populations available in the literature and the one from Campinas. Intervals were calculated using Wilson's method for a binomial distribution (Brown *et al.*, 2001) and a 95% confidence level. A. Montenegro *et al.*, 2005; B. Pool *et al.*, 2006; C. Watts *et al.*, 2009; D. Ventura *et al.*, 2012.

Location		Exact Estimate	Minimum Estimate	Maximum Estimate
Campinas	2016	0	0	1.5
Uganda	2007	2.6	0.5	13.5
Recife	2009	0	0	13
Recife	2003	2.3	0.6	5.8
Rio de Janeiro	2008	1.7	0.01	9.91
USA/Mexico	2005	1.3	0.2	6.8
Salvador	2010	9.6	4.5	18.8
Salvador	2009	12.5	3.5	31.8
Salvador	2008	17.7	13.2	23.2

3. ARTIGO 2: Canibalismo em *Drosophila* é comum e adaptativo

Artigo em preparação para submissão.

CANNIBALISM IN *DROSOPHILA*: WIDESPREAD AND ADAPTIVE

Cesar Henrique Mondini[1,*] and Louis Bernard Klaczko[1]

[1]Department of Genetics, Evolution, Microbiology and Immunology, Institute of Biology, State University of Campinas – UNICAMP, 13083 – 970, Brazil

[*]cesarmondini@hotmail.com

3.1. ABSTRACT

Drosophila can be considered one of the most important model organisms in science, and although major advances in Biology were made using the world's most famous fly, one question remains poorly understood: how and what does it feed on? This is made blatantly evident by the relatively recent and yet not complete understanding of cannibalism in this genus, let alone in *Drosophila melanogaster* itself. In this study we investigated the ubiquity and other aspects of cannibalism in *Drosophila* using seven species spread in the taxon. Through direct observation, we found evidence of cannibalization performed by larvae on other larvae and adults for all tested species, in different degrees, and most of them also cannibalized eggs. Our data also suggests that cannibalization of eggs is directly proportional to time exposed to cannibals and larval density in *D. melanogaster*. Furthermore, we were able to raise into adulthood individuals of both *D. melanogaster* and *Drosophila simulans* when the nutritional input, apart from sugar, was solely available through competitive cannibalization of simultaneously hatched conspecifics. Finally, using artificial directional selection of larval size under nutritional deprivation, we were able to obtain lineages that engaged in cannibalism with different rates. Altogether, this shows that cannibalism is widespread, that it is an important feeding mechanism for *Drosophila,* that it is adaptive under nutritional deprivation, and that it has a genetic backbone which can be selected via larval size. These results provide insight into how competition is dealt within the group, or how infectious diseases may spread in it. Cannibalism can be expected to be performed by larvae in all other stages of development, and it can be under selective forces as it sems to be a relevant source of nutrients in oviposition sites, which tend to be overcrowded.

3.2. INTRODUCTION

Drosophila melanogaster is, arguably, the most studied animal species in the world, and one of the most studied organisms, in general. Given its small size, short reproductive cycle, easy handling and cheap maintenance, it is a model organism for many research areas, such as immunology, genetics, physiology, pathology, and behaviour [1][2][3][4][5], its importance is shown in the fact that 6 Nobel prizes were given to researchers that worked with *D. melanogaster.* But *D. melanogaster* is not the only *Drosophila* that is paramount to science. Population genetics reshaped Biology and Genetics, and to some extent the Natural Sciences as a whole, by providing a mechanism with testable hypotheses for the evolutionary theory. Keystone studies of experimental population genetics research were developed using other species from the *Drosophila* genus [6][7], which are still commonly used in evolutionary research [8][9][10]. However, despite the abundance of studies that use *Drosophila* as a tool or a model, the comprehension of the Natural History of the genus itself is, in a considerable degree, lacking. This is made evident when current research is revealing important basic characteristics of these flies, as their anatomy, interactions, movement, diet, and habitat [11][12][13][14][15]. It is striking that a group of organisms that has been intensively studied for over a century has basal traits of its ecology still being learnt and published.

Cannibalism is an ecological trait that is found in several animal species, with profound evolutionary impacts [16]. Cannibalism can have populational effects that range from enhancing sibling fitness when food is scarce [17] or enhancing male fitness when it is cannibalised by a sexual partner [18], to promote invasiveness of species newly introduced to new habitats [19], as well as being a mechanism for controlling infectious diseases [20], and many others. Given the importance of such behaviour, the literature on the genetics of cannibalism is considerably large. There are many heritability estimates, characterisation of populations or strains and, most of all, mathematical models on the adaptive potential and constraints of cannibalism. However, as far as we are aware, empirical research on selection of cannibalistic behaviour is reduced to few studies: one paper selected a cannibalistic strain of *D. melanogaster* [21] and many studies on hens [22] that selected strains that would not engage in cannibalism since it is a cause of economic loss in hen husbandry.

Comprehending how a behaviour with such a strong impact on fitness can be produced by natural selection is key to fully realising its influence in populations.

Cannibalism exists in *Drosophila* and it is largely overlooked, considering both the importance of the behaviour and the importance of the genus. The first peer-reviewed work on cannibalism in *Drosophila* was published in 1990 [23] and verified that eggs of 7 species (*D. melanogaster*, *Drosophila hydei, Drosophila robusta, Drosophila immigrans, Drosophila putrida, Drosophila tripunctata*, and *Drosophila neohydei*) would fully develop into adults when conspecific adult carcasses were provided as their only source of food. Only 14 years later, in 2004, another investigation on cannibalism of *Drosophila* was reported, and found no evidence of cannibalism being performed by adults [24]. Then, summarising recent findings on cannibalism in *D. melanogaster*, we have learnt that: 1) larvae can cannibalize eggs [25], other larvae [21], and adults [25]; 2) adults can cannibalize larvae that are punctured [25]; 3) cannibalistic behaviour seems to be driven by food deprivation [25]; 4) individuals are able to develop into adults when they are provided a conspecific larval [21] or egg diet [25]; 5) egg cannibalism is more likely to happen with non-related individuals [26]; 6) females protect eggs from cannibalism with pheromones that prevent their consumption by conspecifics [27]; 7) the tendency to cannibalize can be selected under nutritionally deprived environments [21]. There has also been found evidence that there is larval cannibalistic behaviour towards pupae in *D. suzukii* [28], but there is evidence that it does not occur in *D. melanogaster* [25]. And finally, cannibalism of adult carcasses by larvae was observed in *Drosophila simulans* [29].

Considering the importance of *Drosophila* and of cannibalism, and that the vast majority of the knowledge on cannibalism in *Drosophila* is restricted to *D. melanogaster,* this work is focused in understanding cannibalism in other species of the genus. To do so, we investigated the existence of cannibalism in seven species, including *D. melanogaster*, for comparison reasons. We examined the occurrence of cannibalistic behaviour of egg, larval and adult prey. Our studies on the cannibalisation of eggs were detailed in cannibalisation of chorion *per se* and cannibalisation of chorion that would enable access to the embryo, as an attempt to elucidate current conundrum regarding egg cannibalisation [25] [27]. We also inspected how cannibalism is affected by the accessibility to the organs of the prey. Furthermore, we used *D. melanogaster* to obtain a further understanding of cannibalism: 1) we know that *D. melanogaster* individuals can fully develop into an adult exclusively by cannibalizing on provided

conspecifics [21] [25], but can they do so in an environment where cannibalism is competitive (i.e. the prey is the other individuals)?; 2) we know cannibalism in *D. melanogaster* can be selected [21], but can we select cannibalistic behaviour if we select it indirectly using size as an indicator of occurrence of the behaviour?

3.3. RESULTS

CANNIBALISM IS WIDESPREAD IN *DROSOPHILA*

Before performing the main assays, we verified that the nail polish and the food dye used did not significantly attract nor repel the larvae. When we compared the number of larvae in contact with the feeding medium, there was no significant difference between the dishes where the medium had nail polish underneath and those dishes who did not (Fig. 1a; Supplementary Table S1). This indicates that nail polish is not affecting the feeding pattern of the larvae. It's noteworthy that the total observed feeding larvae was much smaller in *D. virilis.* When we compared the number of larvae with coloured gut, there was a significant difference between the group that had the food dye mixed into the feeding medium and the group that had food dye mixed into a block of agar (Fig. 1b; Supplementary Table S2). This indicates that food dye is not attracting nor repelling larvae, differently from food, which is a strong attractant (on average, 51.0 ± 24.4% of the larvae fed of the coloured food dye, when only 8.1 ± 11.7% fed of the coloured agar block). It's important to point out that this difference was not significantly perceived in *D. virilis*, although the same trend of feeding more on the coloured medium was still observed, but in a smaller degree.

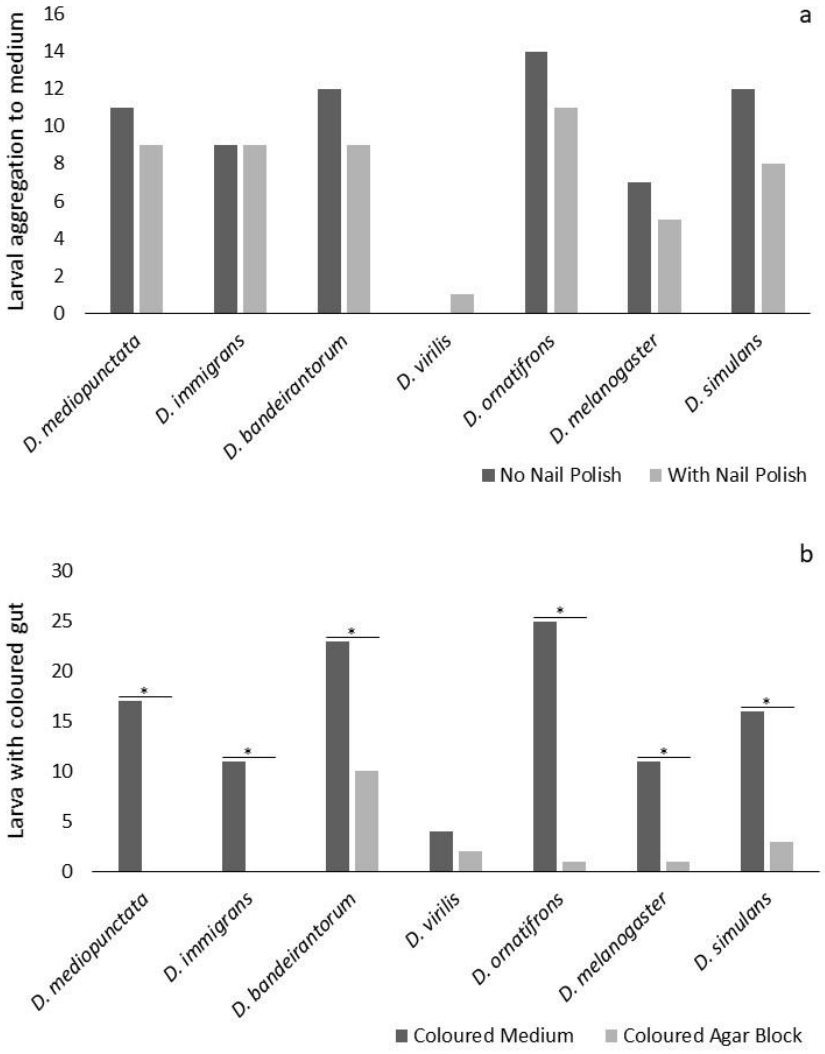

Figure 1. Food dye and nail polish do not affect feeding behaviour. a) For each species, a total of 30 individuals were allowed to feed on medium over nail polish or medium placed directly on agar. The number of larvae feeding on medium over nail polish or agar is not statistically different (Fisher Exact Test; Supplementary Table S1). b) For each species, a total of 30 individuals were allowed to feed on coloured medium or a coloured block of agar. The number of larvae with coloured gut (from the food dye) was statistically greater in the group that had coloured medium available (Fisher Exact Test; Supplementary Table S2).

* Indicates significant difference (i.e. $p < 0.05$).

ADULTS AND LARVAE ARE CANNIBALISED BY LARVAE IN ALL TESTED SPECIES

We observed all the tested species engaging in cannibalism of both adult and larval prey in different degrees (Fig. 2a and Fig. 2b.).

We verified that available prey (not enamelled prey & punctured prey) statistically made larvae aggregate to cannibalise in most tested species, for both types of prey (Fig. 2c and Fig. 2d), if compared to unavailable prey (enamelled prey). Numerically, when we averaged the aggregation among all species, except *D. virilis*, we obtained very similar values for larval and adult prey: 4,34 aggregated larvae on not enamelled larva and 10 aggregated larvae on punctured larvae; 3,83 aggregated larvae on not enamelled adults and 10,17 aggregated larvae on punctured adults (Supplementary Tables S3 and S5). The only species that didn't present a statistically significant difference in aggregation for either adult or larval prey was *D. virilis.* However, in every species, for both types of prey, when it was possible to consume the prey (not enamelled & punctured *versus* enamelled prey), the number of potential cannibals in direct contact to the prey was greater (Supplementary Tables S3, S4, S5 and S6).

This high aggregation around accessible prey suggests that larvae of the tested *Drosophila* species can detect the presence of conspecifics to feed on them. To further examine this hypothesis, we performed a linear regression on the data of aggregation of cannibalising individuals for the three progressive levels of access to the prey (Supplementary Table S7). We detected a positive slope for all species (*i.e.* the easier to access/detect, the higher the number of cannibals). The regression is statistically significant and with high R^2 for all species, except for *D. virilis*, when cannibalisation of larval and adult prey are taken together to evaluate larval detection of prey (Supplementary Fig. S1 and S2, and Supplementary Table S7). These slopes indicate that indeed aggregation to the prey is an accurate measurement of cannibalism, given that almost no individuals were in direct contact with the prey when the prey was enamelled (and, therefore, was inaccessible to be eaten). Another result from the slopes is that for conspecific prey in general, most species (exception is *D. virilis*) will engage in cannibalism in higher degrees if the prey is easier to access. This suggests that cannibalism in these species is more affected by accessibility then by lack of food itself, which indicates that feeding on conspecific carcasses must be more common than cannibalistic predation. It's noteworthy, though, that more regressions

were significant for larval than for adult prey, indicating that cannibalisation of larvae can be a more established behaviour than the one of adults.

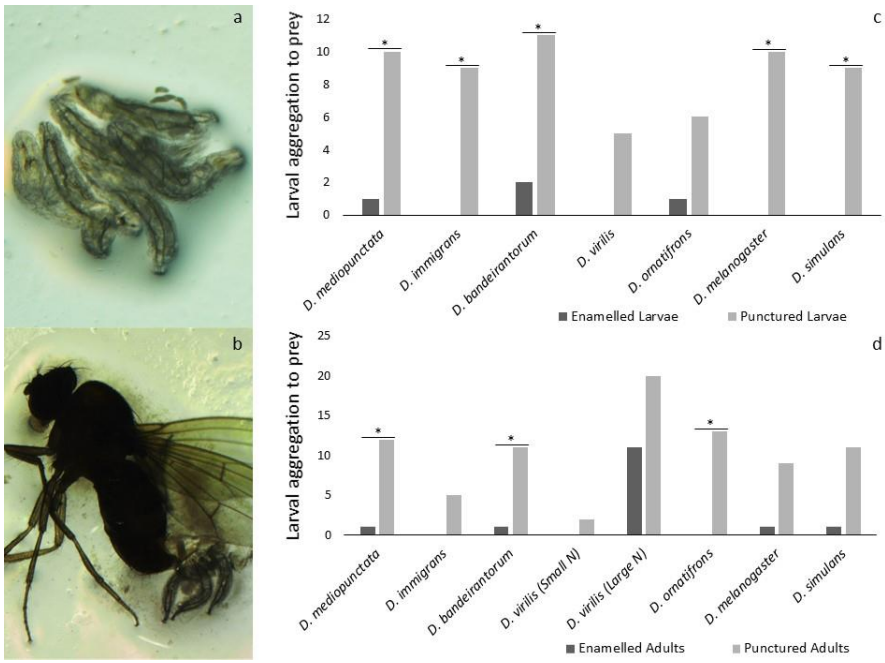

Figure 2. Larval cannibalism on adults and larvae in different degrees of access. a) Picture of cannibal larvae feeding on a dead conspecific larva which was punctured (*D. melanogaster*). b) Picture of cannibal larvae feeding on a dead conspecific adult which was punctured (*D. bandeirantorum*). c) Comparison of the number of potential cannibals in contact with the larval prey between enamelled and punctured prey. The number of larvae in direct contact with the prey was statistically greater, for most species, when the prey was accessible (Fisher Exact Test; Supplementary Table S4). d) Analogous comparison to the one before, but using adult prey. The results are also analogous, given that most species had a statistically greater number of larvae in contact to the prey that as accessible (Fisher Exact Test; Supplementary Table S6). Here, we performed the essay in 2 different sample sizes for *D. virilis* (Small N = 30 potential larvae; Large N = 90) because we hypothesised that the phenomenon could require a greater sample to be detected, but both assays were not statistically significant.

* Indicates significant difference (i.e. $p < 0.05$).

For adult prey, the number of individuals engaging in cannibalism was greater in every species than it was observed in *D. melanogaster*, except for *D. virilis* and *D. immigrans*. On the other hand, for larval prey, except for *D. virilis* and *D. ornatifrons*, which had fewer cannibals, all species presented similar number of individuals cannibalising on larval prey.

CHORIONS ARE CANNIBALISED BY LARVAE IN MOST TESTED SPECIES

Except for *D. bandeirantorum* and *D. virilis*, all other species presented consumption of conspecific chorion (Fig. 3a). When starved larvae were presented to the possibility of feeding on conspecific eggs which had had their chorions coloured with food dye, we observed, in 5 out of 7 tested species, individuals with coloured gut, which indicates cannibalism of the outer layer of the egg (Supplementary Table S8). It does not indicate whether the embryo itself was cannibalised because the protection of the chorion prevents the embryo itself from being stained. Although cannibalism was present in most species, it varied in degree between species, ranging from 5% of the individuals to 60% of the individuals engaging in cannibalistic behaviour (Fig. 3c). The only species that showed a greater number of individuals cannibalising chorion than *D. melanogaster* was *D. simulans*.

CANNIBALISM BY LARVAE MADE EMBRYOS AVAILABLE TO CONSUMPTION IN D. MELANOGASTER *AND* D. SIMULANS

Given that the previous assay only assessed the consumption of chorion, we further investigated whether consumption of embryo, supposedly the more nutrient rich part of the egg, could be made possible via cannibalism of the egg that would perforate the chorion. We used only the three species that engaged more in cannibalism of chorion in the previous assay (*i.e. D. mediopunctata*, *D. melanogaster* and *D. simulans*), analysing the integrity of the chorion in different arrays of density of potential cannibal larvae and time spam in which the eggs were made available (Fig. 3b). In all three species, there were eggs whose chorions were cannibalised until exposure of the embryo (Fig. 3d). Nonetheless, when time was reduced and the density of individuals was small, we observed very few ruptured chorions, and we used this as a control for a statistical test to verify the influence of time and larval density in rupturing chorions via cannibalism (Figure 3d; Supplementary Table S9). *D. simulans* was consistently non-significant neither for time nor for larval density, but both factors

increased the absolute number of eggs that had exposed embryos. *D. melanogaster* had a significant influence of both ($p < 0.0001$ for both time and larval density) in the rupturing of chorions and therefore exposure of embryo. *D. mediopunctata* only had a significant greater number of violated chorions when larval density was low but time spam was large ($p = 0.0024$).

It is paramount to mention that all eggs whose chorions were violated had, upon visual inspection, intact embryos (*e.g.* Fig. 3b). Thus, although we could detect cannibalisation of eggs up to a point of chorion rupture, we have no evidence that the available embryos were in fact cannibalised.

EGG TO ADULT DEVELOPMENT VIA COMPETITIVE CANNIBALISM

Cannibalism is a behavioural mechanism that enables individuals from *D. melanogaster* and *D. simulans* to develop from eggs into adults in an exclusively competitive cannibalistic diet. In environments where only water and sugar were available, the number of conspecific eggs were critical for the survival and development of individuals up to the adult stage. For both species, when 25 eggs (40 replicas; 1000 individuals total) where placed in the same dish, zero individuals developed into adults. However, when 100 eggs (10 replicas; 1000 individuals total) where placed in similar dishes, both species had individuals that developed into adults (Supplementary Tables S10 and S11). Out of the 10 dishes assembled with 100 eggs (*i.e.* with a greater nutritional input than dishes with 25 eggs) for each species, 1 (*i.e.* 10%) presented one fully developed adult of *D. melanogaster,* and five (*i.e.* 50%) presented a fully developed adult of *D. simulans*. In other words, among the 100 egg-dishes, one out of 1000 individuals developed into adulthood in *D. melanogaster*, and five out of 1000 did the same in *D. simulans*. One of the dishes with adults of *D. simulans* presented a second pupa that didn't hatch. Cannibalism seems to be a vital behaviour in situations where nutrients are very limited and larval density is extreme for these species of *Drosophila*. The fact that many more individuals of *D. simulans* developed into adults than *D. melanogaster* is expected since cannibalism of egg chorion in *D. simulans* is more expressive than in *D. melanogaster* (Fig. 3c).

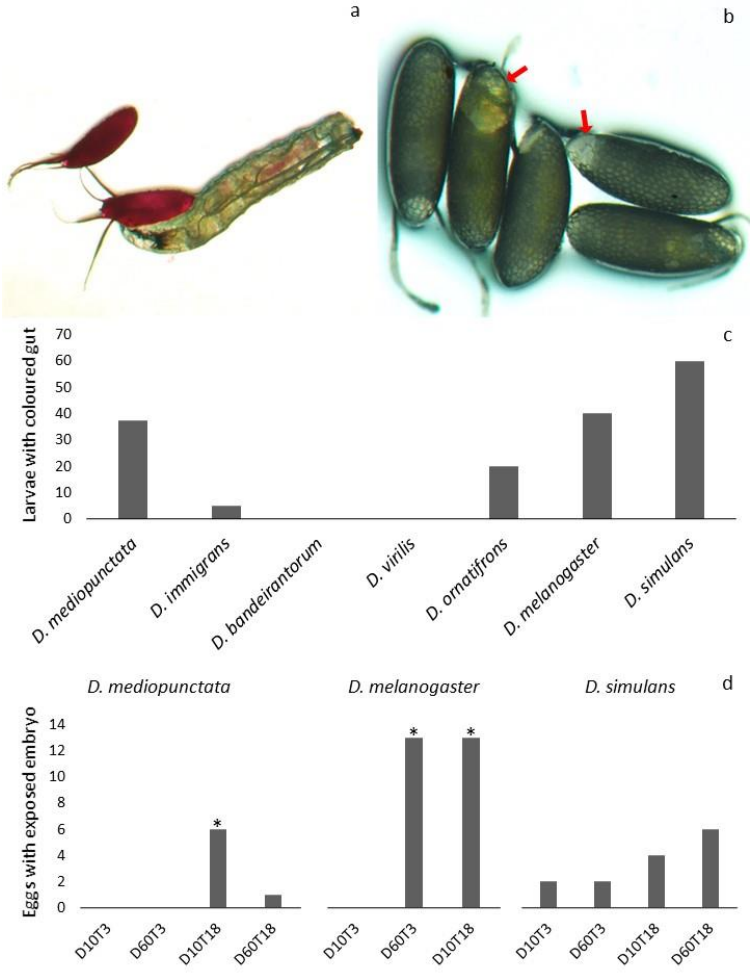

Figure 3. Larval cannibalism of eggs. a) Larva of *D. melanogaster* cannibalising egg chorion; we can observe the presence of food dye in the gut of the larva. b) Eggs of *D. melanogaster* after being cannibalised by larvae. Red arrows indicate regions where the chorion was cannibalised up to exposure of the embryo. c) Number of larvae with coloured gut after eggs coloured with food dye were made available (Supplementary Table S8). d) Number of the eggs that were made available to cannibalisation that presented any type of visible rupture in the chorion, thus making the embryo available.

* Indicate statistically significant comparison between the group tested and the D10T3 (Low density & Short time spam) which was used as a control. Fisher Exact Test (Supplementary Table S9).

CANNIBALISTIC BEHAVIOUR CAN BE INDIRECTLY SELECTED

After applying directional artificial selection for body size of larvae under nutritional deprivation, in both directions, we verified that cannibalism has a genetic background and can be selected indirectly. Larger larvae (*i.e.,* those that more likely had engaged in cannibalism) were used to produce Pool 3+, but random individuals of this strain were not statistically larger than those from Pool 2- (strain produced from smaller larvae; *i.e.* those that less likely had engaged in cannibalism) (Fig. 4a; Supplementary Table S12). Its noteworthy that although average size did not differ statistically, the standard deviation of Poll +3 was greater than the one of Pool 2- (approximately 0,3 and 0,2, respectively) These findings indicate that selection of body size did not select body size itself, because larger larvae under nutritional deprivation that favoured cannibalism did not produce larger offspring, nor did smaller larvae produce smaller offspring. Actually, the data suggest that what have been selected is not larval size, but behaviour, because larvae from Pool 3+ engaged more in cannibalism than larvae from Pool 2-, statistically (Fig. 4b; Supplementary Table S13; Fisher's Exact Test: $\alpha = 0.05; N = 400;$ $p < 0.0001$). This suggests that larger individuals in a nutritionally deprived scenario are larger because they are prone to engaging in cannibalism more often. This tendency can be inherited and selected, which is itself evidence that cannibalistic behaviour has a genetic underlaying controlling mechanism.

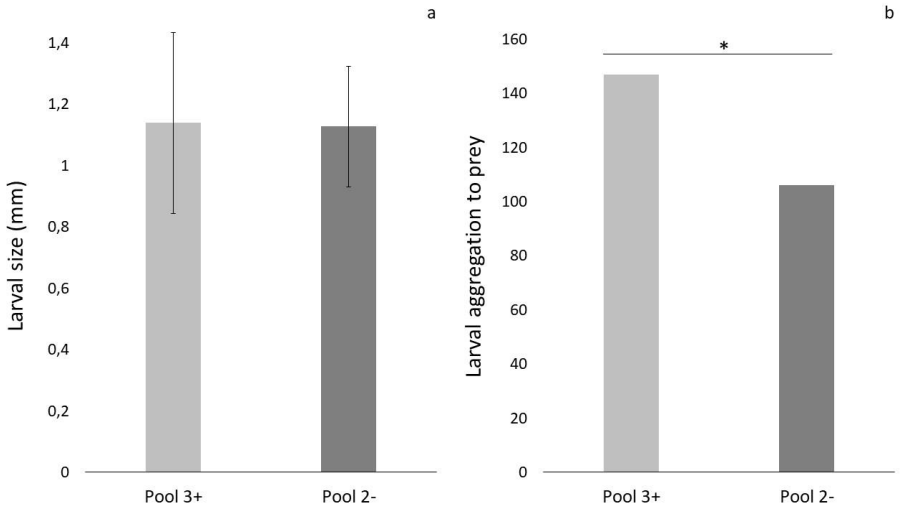

Figure 4. Experimental evolution of cannibalism via indirect selection. a) Average larval size of random individuals from Pool 3+ and Pool 2-, three days after oviposition. Bars represent standard deviation. Average sizes are statistically not different (Supplementary Table S12). b) Number of larvae in direct contact to prey for Pool 3+ and Pool 2-. The contact with the prey was used to evaluate occurrence of cannibalistic behaviour. More larvae from Pool 3+ engaged in cannibalism ($p < 0.0001$, Fisher Exact Test, Supplementary Table S13). Thus, cannibalism can be considered more often in Pool 3+, providing evidence it can be selected indirectly.

* Indicates significant difference (i.e. $p < 0.05$).

3.4. DISCUSSION

Our results demonstrate that, in different degrees, larvae from all seven tested species cannibalize other larvae and adults (Fig. 2). Also, except for *D. virilis* and *D. bandeirantorum*, all other species cannibalize egg chorion (Fig. 3c). Considering that these species are phylogenetically disperse inside the genus *Drosophila* [30], these findings indicate that cannibalism performed by larvae is widespread in the group and potentially very common in specific scenarios. Although cannibalism is an evolutionary complex interaction that is dependant of many factors, theoretical work as well as observational studies have shown that, unless there are high costs to cannibalizing [16], cannibalism is beneficial both at a populational and at an individual level, and it is possible to be evolutionarily maintained in situations in which food is scarce or overcrowding is common [31] [32]. These benefits of cannibalism when food is scarce are made evident in the experiment where we obtained adults exclusively via competitive cannibalism (Supplementary Tables S10 and S11), because, differently from previous work [21] [23], in our assay the individuals had to compete among themselves, cannibalizing each other to survive. This experiment encompasses a situation in which the lack of food, which was virtually inexistent apart from competing conspecifics, would be fatal to every individual. But this deprivation was overcome through cannibalistic competition, that enabled the survival of one individual. Considering that the oviposition sites of *Drosophila* in nature are usually fruits in which many individuals of many species compete [33], and that oviposition by a female elicits oviposition by other females over time [34], the sites where larvae of *Drosophila* develop are commonly overcrowded, leading to lack of food, which in turn enhances the evolutionary benefits of cannibalism.

But, beyond overcrowding, there are other factors that could favour cannibalistic behaviour in such a wide range of species as the one in which we detected cannibalism in this work. First, the progressive oviposition of conspecifics [34] creates an age-structured population that would enable few older larvae being predatorily cannibalized by many younger larvae [21], and these younger larvae are the ones who would experience higher crowding, which in turn represent a harsher environment. Theoretical work provides evidence that cannibalism in low frequencies, in early developmental stages, and in nutritionally deprived situations can provide great positive effects in the life history of individuals [16]: the lifeboat hypothesis.

Furthermore, this age gap would mean that individuals are more likely to predate non-sibling conspecifics, which reduces the evolutionary costs of cannibalism [16]. This cannibalization of non-siblings would be reinforced by chemically induced repulsion that females provide by coating their eggs with pheromones [27], and the fact that, even without such pheromones, individuals cannibalize non-sibling preferably [26]. This intricate set of conditions provide a solid reason for cannibalism to have been observed in so many species as we have. Taken all into account, observational studies, in natural conditions, of rates of cannibalism of *Drosophila* are still necessary to fully comprehend the importance and extent of this behaviour.

It would be interesting to verify whether the different rates of cannibalism that we observed for different species are directly proportional to the crowding levels that such species find in natural developmental sites. Almost the entirety of the knowledge on cannibalism in *Drosophila* was obtained using *D. melanogaster*, but our results suggest that *D. melanogaster* engages in cannibalism in different rates than other species (Fig. 2). Considering the smaller rates of cannibalism that were observed in *D. virilis* and *D. ornatifrons* regarding larval prey (Fig. 2a), it would be interesting to test whether in nature these species develop in environments in which larval density is lower than that the other tested species. This is a testable hypothesis for the theoretical application of the life boat hypothesis to *Drosophila* [16]. In another perspective, the rates of cannibalisation of chorion in different species, which is very variable (Fig. 3c) could be compared with rates of production of non-viable eggs and the production of repellent pheromones [27] to evaluate the payoff of cannibalising siblings.

The cannibalization of eggs has very specific costs and benefits. There is certain discussion regarding the frequency or existence of egg cannibalism [25] [26] [27] [28] and we present results that help elucidate this conundrum, mostly by corroborating the data that indicate that pheromones in the wax layer would prevent cannibalism of eggs [27]. All species we studied showed consumption of chorion (Fig. 3a), but the pattern of consumption of chorion that made the embryo accessible was very inconsistent (Fig. 3b). The eggs we used were frozen before the assays, which could provide a mechanism to disrupt the repulsive wax layer. For three species, in which we observed exposure of embryos, time and density showed positive influence over the exposure of conspecific embryos. Both factors are elements which would facilitate the rupture of the protective layer and enable cannibalism of eggs fully. But even after being frozen, no actual embryo was consumed in our assays, reinforcing

the idea that the wax layer can deter cannibalism. Hence, in the environment larvae of Drosophila usually develop, with a high density of individuals, there is evidence that eggs would initially be protected from cannibalism; but, if the eggs don't emerge rapidly, there is a continuously growing chance the wax layer would be disrupted and the egg fully cannibalized. This system would be specifically advantageous, from an evolutionary perspective, for species that lay a significant number of non-viable eggs [36] or that produce non-viable eggs due to infection [37], because such eggs would then provide nutrition to relatives. Furthermore, although cannibalism was enough to rupture the chorion, the vitelline membrane resisted in our assays, which indicates that the comprehension of egg cannibalism in *Drosophila* is a two stage investigation, and the second, regarding the rupture of the vitelline membrane remains unclear.

The congruent positive slopes of the curves of larvae in contact with prey (Supplementary Fig. S1 and S2) expose an interesting possible aspect of cannibalism in *Drosophila*: it might be non-intentional. As mentioned before, natural developmental sites of *Drosophila* are usually crowded with many individuals, of many species, in many developmental stages. Some *Drosophila* species have been shown to possess social [23] and external [29] digestion, and to feed on many different sources of food [25] [29]. Recent research has in fact observed that, despite *D. melanogaster* being considered saprophagous for a long time, feeding mainly on the decomposed produces of yeast, when food from animal origin and yeast are consumed simultaneously, individuals develop faster and achieve a greater body-size [29]. Moreover, it was shown that larvae would prefer to feed on this combination than on either of the individual components. Having these facts in mind, individuals that for any given reason would die in developmental sites would be digested by the remaining larvae and provide an advantage to the conspecifics, but these remaining ones not necessarily would be purposefully feeding on conspecifics. This evolutionary advantage of cannibalism would exist without the typical payoff of cannibalism: aggression and reduction of inclusive fitness as a result of predation. Differently from the lifeboat hypothesis, this mechanism would be just a beneficial by-product of the voraciousness and feeding habits of larvae. The fact that, for every tested species, more individuals cannibalized on the punctured larval or adult prey (Supplementary Tables S3 and S5) is an indication of the non-specificity of cannibalism, which in this case was more limited by the easiness of access to internal organs and ability to detect chemical cues [21] than whether the food source is a conspecific or not.

Nevertheless, the extent of the purposefulness of cannibalism become less blurred when the results of our experimental evolution assay are taken into account (Fig. 4), because we were able to both positively and negatively select cannibalism separately from general feeding habits. Using selection, we were able obtain strains that would engage in cannibalism in different rates (Fig. 4b), which is evidence of a genetic basis for such behaviour, but that was achieved using body size instead of actual observation of cannibalism. The use of body size as evidence of cannibalism is reasonable given that the context that the individuals were in allowed only cannibalism as a source of food intake. But body size in fact is a direct indicator of capacity and/or frequency of feeding. It is known that the *for* gene and the PI3K/Akt pathway affect foraging behaviour and body size and growth in *D. melanogaster*, and that wider foraging patterns and larger body sizes are selected when larvae develop in high-density environments [35]. Therefore, one could argue that this general feeding behaviour is what was selected, and cannibalism was just a fortuitous result that was dragged along body size. However, despite the fact that the differently selected strains exhibited different rates of cannibalism, the larval size of these selected strains was statistically the same (Fig. 4a). This discards the hypothesis that what was selected was a general feeding pattern like the *for* gene or the PI3K/Akt pathway, because, if this were the case, the individuals would present different body sizes as well. Specific analyses of gene expression in cannibalistic and non-cannibalistic scenarios are needed to determine which specific pathways are involved in cannibalism and how connected these cannibal-related pathways interact with the more general *for* gene or the PI3K/Akt pathway.

Our findings show that cannibalism in *Drosophila* larvae is widespread inside the genus and it is expressed in consumption of every stage of the lifecycle of the flies. Varying in degree of manifestation, the cannibalistic behaviour is consistent for larval prey in the studied species, with a greater range of expression for adult prey and an even more wide-ranging one for egg prey. These fluctuations are most likely to be associated with the life history of each species, specifically connected to the larval development site, larval density and food availability. We also showed that cannibalism is adaptive in food-deprived environments and that it has genetic bases that are in part independent from general feeding regulatory mechanisms. Taken together, these results expose the importance of cannibalism as a well stablished behaviour in *Drosophila.*

3.5. METHODS

LINEAGES

Unless where differently specified, we used flies from laboratorial collections of *D. melanogaster* (Canton-S)*, D. simulans, D. ornatifrons* (CAE 221)*, D. virilis* (REC)*, D. mediopunctata* (ITC 229 ET)*, D. immigrans* (BTA 212) and *D. bandeirantorum* (ITI B9). *D. simulans* and *D. melanogaster* were raised on standard cornmeal-molasses medium at 23ºC, all the others were raised on flour-milk medium at 16ºC, except for *D. ornatifrons* which was kept at 20ºC.

ACQUISITION OF EGGS AND POTENTIAL CANNIBALS

Eggs

Eggs were collected whether to be used as prey or to obtain potential cannibal larvae for the assays, both of which require a very homogeneous age. Therefore, the collection was held for no longer than 4 hours. Adults were placed inside bottles with glass slides covered in a water-agar-sugar mixture (1.5% m/v agar; 4% m/v sugar) topped with dried yeast (*Saccharomyces cerevisiae*) to attract ovipositing females. After 4 hours the slides were removed and the eggs inside the agar were transferred either onto a Petri dish for cannibalisation (as potential prey) or for development under extreme nutritional deprivation (as potential cannibals). Both dishes held the same water-agar-sugar mixture aforementioned.

Potential Cannibals

Potential cannibals were allowed to develop for 3 days for *D. simulans* and *D. melanogaster*, 4 days for *D. virilis* and 5 days for the others. This time spam was based on *D. melanogaster* [25] and adapted to the other species, aiming to have the larvae as nutritionally deprived as possible but still considerably healthy and mobile.

CANNIBALISTIC ASSAYS

The potential cannibal larvae obtained were used in a series of assays to verify whether cannibalistic behaviour was to be expressed in different species and towards different types of prey. All essays were performed using 35mm plastic Petri dishes with water-agar-sugar mixture (1.5% m/v agar; 4% m/v sugar) and covered with micro-punctured Parafilm® to prevent larvae from escaping while allowing ventilation. After a period of 3 hours (unless specified otherwise) different measures were taken. Unless when specified differently, all 7 species were used.

Preliminary tests

Some of the assays used food dye or nail polish. To guarantee that these substances would not affect the aggregation of potential cannibal larvae around the prey we performed two assays. For the food dye, Control group consisted of 3mm³ of raising medium, whereas Treatment group consisted of 3mm³ of solid agar solution, both made with 5% food dye and in triplicates; 10 larvae were placed on each dish, and after 3 hours the number of larvae with coloured gut was counted. For the nail polish, Control group consisted of 3mm³ of raising medium placed directly on the agar solution of the dish, whereas the Treatment group consisted of the same medium but with a layer of nail polish in between the medium and the agar solution, both in triplicates; 10 larvae were placed in each dish, and after 3 hours the number of larvae in direct contact with the raising medium was counted.

Adults as prey

To test the occurrence of cannibalism through aggregation around the prey, 10 potential cannibal larvae and 1 conspecific potential adult prey were placed in each plastic Petri dish. The potential adult prey was first euthanized using low temperature and afterwards used in the assays encompassing three treatments: Enamelled (fully covered with nail polish), Control (no further modification), and Punctured (drastically ruptured using a needle). Each treatment was assembled in triplicate for each tested species. The three treatments composed a gradient of easiness of access to the internal organs of the potential adult prey. At the end of 3 hours the number of larvae in direct contact with any body part of the prey was counted and whether individuals were feeding in conspecifics was observed.

Larvae as prey

This experiment is analogous to “Adults as prey” as regards preparation, times, treatments and measures, but differs in that in this assay the potential prey used was a euthanized conspecific wandering-state larva [21]. This was used to provide insight whether cannibalistic behaviour would occur over larvae.

Chorion as prey

To investigate whether potential cannibals could consume chorion, conspecific eggs which were to be used as potential prey were washed with isopropanol, chloroform, and ether. Ether was allowed to evaporate, and the eggs were placed into 5% food-colouring solution (Junco®, composed of erythrosine and ponceau 4R) at 20ºC overnight [38] [39], which allowed the food dye to stain the inner layers of the chorion. Each Petri dish was assembled using 10 potential cannibal larvae and 5 coloured eggs, and for each species the test ran in quadruplicate. After 3 hours, the number of larvae with coloured gut, which would indicate chorion cannibalisation, were counted and photographed. The same was done with the eggs, which were thoroughly examined with a zoom stereo microscope to inspect for ruptures.

Embryo exposure via consumption

To examine whether potential cannibal larvae would be able to access the nutrients of conspecific embryos, therefore crossing the protective layer of the chorion, we used *D. melanogaster*, *D. simulans*, and *D. mediopunctata*. We placed 5 eggs, which were not processed except for euthanization through low temperature, in each of the plastic Petri dishes. Density of potential cannibal larvae and the time they were able to access the prey composed a set of 4 treatments: $D^{H}T^{H}$ (60 larvae, 18 hours), $D^{H}T^{L}$ (60 larvae, 3 hours), $D^{L}T^{H}$ (10 larvae, 18 hours), and $D^{L}T^{L}$ (10 larvae, 3 hours). For every species and treatment, the experiment was conducted in six replicas. The number of ruptured chorions (which exposed the embryo) per Petri dish was counted after inspection under a zoom stereo microscope.

DEVELOPMENT ASSAY

D. melanogaster and *D. simulans* were used to test the hypothesis that eggs could fully develop into adults in a highly nutritiously-deprived environment where most nutrients would obligatorily be acquired via competitive cannibalism. Such an environment was achieved by placing eggs into Petri dishes with water-agar-sugar mixture (1.5% m/v agar; 4% m/v sugar) which provided the emerged individuals with water and sugar only.

Petri dishes (35 mm) were set with 100 (10 replicas, 1000 eggs total) or 25 eggs (40 replicas, 1000 eggs total) for each species. The number of eggs represented two treatments: High and Low availability of nutrients accessible through competitive cannibalism, respectively.

Petri dishes were then covered with Parafilm® which was, in turn, punctured with a fine needle, in order to allow gas exchange but inhibit larvae from escaping. All replicas were kept in a stable 23ºC environment for 30 days. After that period the number of pupae and adults in the dishes were counted, as well as the presence of fungal growth over the agar and the occurrence or not of excavation into the agar by the larvae were verified.

INDIRECT ARTIFICIAL SELECTION OF CANNIBALISTIC BEHAVIOUR

We tested whether cannibalistic behaviour could be selected indirectly. In a nutritiously deprived environment, cannibalism can be a mechanism to acquire nutrients and, therefore, individuals with a greater chance to exhibit cannibalistic behaviour would develop faster and be bigger.

Population setup and artificial selection

We crossed six of our lab lineages of *D. melanogaster* (Canton-S, Wild, 8616, 1615, 1936, RED 85) in a series of combinations to produce a population with high genetic variability which we named “Pool”. We allowed females from Pool to oviposit on a water-agar-sugar mixture (1.5% m/v agar; 4% m/v sugar) over glass slides for 3 hours, to ensure uniformity in hatching. Larvae were then collected from

these slides, which only provided water and sugar, after three days, using body size as criterium.

From Pool we produced Pool 1+ (created with larger larvae from Pool) and Pool 1- (created with smaller larvae from Pool). Then, from the larger larvae from Pool 1+, we produced Pool 2+ and, from that, Pool 3+. Similarly, from the smaller larvae from Pool 1-, we produced Pool 2-.

Larvae from Pool 3+ and Pool 2-, after being kept for three days in a water-agar-sugar mixture (1.5% m/v agar; 4% m/v sugar) dish, were preserved in a 1:1 ethanol methanol solution, photographed and measured in length.

Cannibalistic assay with populations that underwent artificial selection

To verify whether selection of body size under nutritional stress would indirectly select propensity of exhibiting cannibalistic behaviour, we used random larvae (potential cannibals) from Pool 3+ and Pool 2-.

Petri dishes (35mm) were assembled using water-agar-sugar mixture (1.5% m/v agar; 4% m/v sugar), one larva prey (wandering-state) and 40 potential cannibals for 2 hours (5 replicas). Cannibalistic behaviour was assessed as a function of aggregation of cannibals to the prey, counting the number of cannibals in direct contact with the prey.

IMAGE ACQUISITION

All images were obtained using a Nikon® DS-Fi2 camera attached to a SMZ 1000 zoom stereo microscope and the software NIS-Elements®.

3.6. ACKNOWLEDGEMENTS

We thank K. A. Carvalho, M. R. D. Batista, F. B. Rocha, R. Cavasini, G. B Passos, R. E. S. Penha and F. Uno for all the input in the development of the work. We thank FAPESP, CAPES and CNPq for funding.

3.7. AUTHOR CONTRIBUTION STATEMENT

Original idea: C.H.M. and L.B.K.; experimental design: C.H.M. and L.B.K.; experimental execution: C.H.M.; data analysis: C.H.M. and L.B.K.; manuscript: C.H.M. and L.B.K..

3.8. ADDITIONAL INFORMATION

Supplementary information accompanies this paper at http://www.nature.com/srep

Competing interests: The authors declare no competing interests.

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3.10. SUPPLEMENTARY TABLES

Species	Control Medium over Agar				Treatment Medium over Nail Polish				Fisher Exact Test N = 60; α = 0.05
Replicate	1	2	3	Total	1	2	3	Total	p-value
D. mediopunctata	4	5	2	11	3	3	3	9	p = 0.7857
D. immigrans	3	3	3	9	4	1	4	9	p = 1.0000
D. bandeirantorum	5	3	4	12	4	3	2	9	p = 0.5889
D. virilis	0	0	0	0	0	0	1	1	p = 1.0000
D. ornatifrons	5	5	4	14	4	4	3	11	p = 0.6010
D. melanogaster	2	1	4	7	3	1	1	5	p = 0.7480
D. simulans	3	5	4	12	5	3	0	8	p = 0.4118
TOTAL				65				52	

Table S1. ***Data and Statistics of nail polish effect.*** The count represents the number of larvae in direct contact with the food, thus feeding. No significant difference was detected between Control and Treatment, indicating that nail polish does not affect feeding in larvae. We used 10 potential individuals, hence each statistical test had N=60. We used Fisher exact test because we had categorical data and some categories had a frequency smaller than 5.

Species	Control Coloured Feeding Medium				Treatment Coloured Agar Block				Fisher Exact Test N = 60; α = 0.05
Replicate	1	2	3	Total	1	2	3	Total	p-value
D. mediopunctata	7	4	6	17	0	0	0	0	**< 0.00001**
D. immigrans	5	5	1	11	0	0	0	0	**0.0003**
D. bandeirantorum	9	7	7	23	1	3	6	10	**0.0016**
D. virilis	0	0	4	4	0	1	1	2	0.6707
D. ornatifrons	7	10	8	25	1	0	0	1	**< 0.00001**
D. melanogaster	5	0	6	11	0	0	1	1	**0.0025**
D. simulans	0	7	9	16	3	0	0	3	**0.0006**
TOTAL				107				17	

Table S2. ***Data and Statistics of food dye effect.*** Significant values in bold. The count represents the number of larvae with coloured gut, *i.e.* which had fed from a compound with food dye. Except for *D. virilis*, all species presented a significant difference between Control and Treatment, indicating that is not the food dye that affects feeding here, but the presence or absence of nutrients. We used 10 potential individuals, therefore each statistical test had N=60. We used Fisher exact test because we had categorical data and some categories had a frequency smaller than 5. The p-values from *D. mediopunctata* and *D. ornatifrons* analyses were inferior to 0.00001, the limit of our Fisher Exact Test.

Species	CANNIBALISM OF LARVAE												
	Treatment 1 Enamelled Larvae				Treatment 2 Intact Larvae				Treatment 3 Punctured Larvae				Visual Observation of Cannibalistic Behaviour
Replicate	1	2	3	Total	1	2	3	Total	1	2	3	Total	
D. mediopunctata	0	0	1	1	1	2	2	5	3	3	4	10	Yes
D. immigrans	0	0	0	0	2	1	1	4	4	4	1	9	Yes
D. bandeirantorum	0	1	1	2	3	1	1	5	5	4	2	11	Yes
D. virilis	0	0	0	0	1	0	0	1	3	2	0	5	Yes
D. ornatifrons	1	0	0	1	1	1	1	3	2	0	4	6	Yes
D. melanogaster	0	0	0	0	1	2	0	3	2	3	5	10	Yes
D. simulans	0	0	0	0	3	0	2	5	2	4	3	9	Yes
TOTAL				4				26				60	

Table S3. ***Data of cannibalism of larval prey.*** The count represents the number of potentially cannibal larvae in direct contact with the larval prey, thus being attracted to feed. In every species the more accessible the prey the higher was the aggregation of potential cannibals. On every species there were individuals that were visually feeding on conspecific carcasses.

Species	*LARVAE - Fisher Exact Test (N = 90; α = 0.0166)*		
	Enamelled X Intact *p-value*	*Intact X Punctured* *p-value*	*Enamelled X Punctured* *p-value*
D. mediopunctata	0.1945	0.2326	**0.0056**
D. immigrans	0.1124	0.2092	**0.0019**
D. bandeirantorum	0.4238	0.1432	**0.0102**
D. virilis	1.0000	0.1945	0.0522
D. ornatifrons	0.6120	0.4716	0.1028
D. melanogaster	0.2370	0.0575	**0.0008**
D. simulans	0.0522	0.3604	**0.0122**

Table S4. ***Statistics of cannibalism of larval prey.*** Values in bold are statistically significant; we performed a Bonferroni correction on the α values, using m = 3. Except for *D. virilis* and *D. ornatifrons*, all species had a significant difference between the enamelled group and the punctured group, the extremes of the nutritional accessibility gradient; this indicates that the presence of readily available larval prey attracted cannibal larvae to feed.

Species	CANNIBALISM OF ADULTS												
	Treatment 1 Enamelled Adults				Treatment 2 Intact Adults				Treatment 3 Punctured Adults				Visual Observation of Cannibalistic Behaviour
Replicate	1	2	3	Total	1	2	3	Total	1	2	3	Total	
D. mediopunctata	0	0	1	1	0	1	2	3	5	3	4	12	Yes
D. immigrans	0	0	0	0	0	0	2	2	3	0	2	5	Yes
D. bandeirantorum	0	1	0	1	1	1	3	5	3	5	3	11	Yes
D. virilis (Small N)	0	0	0	0	0	0	2	2	1	0	1	2	Yes
D. virilis (Large N)	1	4	6	11	6	2	6	14	5	8	7	20	Yes
D. ornatifrons	0	0	0	0	0	0	4	4	4	5	4	13	Yes
D. melanogaster	0	1	0	1	0	3	0	3	5	3	1	9	Yes
D. simulans	0	0	1	1	0	1	5	6	4	5	2	11	Yes
TOTAL				15				39				83	

Table S5. ***Data of cannibalism of adult prey.*** The count represents the number of potentially cannibal larvae in direct contact with the adult prey, thus being attracted to feed. In every species the more accessible the prey the higher were to aggregation of potential cannibals. On every species there were individuals that were visually feeding on conspecific carcasses. There are two sampling groups of *D. virilis*: “Small N” had 10 potential larvae per replicate; “Large N” had 30 potential larvae per replicate.

Species	ADULTS - Fisher Exact Test (N = 90; α = 0.0166)		
	Enamelled X Intact p-value	Intact X Punctured p-value	Enamelled X Punctured p-value
D. mediopunctata	0.6120	0.0153	**0.0011**
D. immigrans	0.4915	0.4238	0.0522
D. bandeirantorum	0.1945	0.1432	**0.0025**
D. virilis (Small N)	0.4915	1.0000	0.4915
D. virilis (Large N)	0.6792	0.3678	0.1318
D. ornatifrons	0.1124	**0.0204**	**>0.0001**
D. melanogaster	0.6120	0.1042	**0.0122**
D. simulans	0.1945	0.1432	**0.0025**

Table S6. ***Statistics of cannibalism of adult prey.*** Values in bold are statistically significant; we performed a Bonferroni correction on the α values, using m = 3. Except for *D. virilis* (both sample sizes) and *D. immigrans*, all species had a significant difference between the enamelled group and the punctured group, the extremes of the nutritional accessibility gradient; this indicates that the presence of readily available adult prey attracted cannibal larvae to feed.

Species	LARVAL PREY				ADULT PREY			
	Slope deviation from zero (F-test)		Goodness--of-fit	Slope	Slope deviation from zero (F-test)		Goodness--of-fit	Slope
	F (1,7)	p-value (α = 0.0166)	R^2		F (1,7)	p-value (α = 0.0166)	R^2	
D. mediopunctata	45.97	**0.0003**	**0.868**	1.50	19.11	**0.0033**	**0.732**	1.83
D. immigrans	14.06	**0.0072**	**0.668**	1.50	3.95	0.0873	0.361	0.83
D. bandeirantorum	11.12	**0.0125**	**0.614**	1.50	18.75	**0.0034**	**0.728**	1.67
D. virilis (Small N)	5.00	0.0604	0.417	0.83	1.31	0.2896	0.158	0.33
D. virilis (Large N)	No Data				3.32	0.1114	0.320	1.50
D. ornatifrons	3.34	0.1102	0.323	0.83	15.50	**0.0056**	**0.689**	2.17
D. melanogaster	15.44	**0.0057**	**0.688**	1.67	4.80	0.0646	0.407	1.33
D. simulans	14.06	**0.0072**	**0.668**	1.50	6.03	0.0437	0.463	1.67

Table S7. ***Data and Statistics of the linear regressions for the assays on cannibalism of larval and adult prey.*** Values in bold are statistically significant; we performed a Bonferroni correction on the α values, using m = 3. Every species studied, for both larval and adult prey, showed a positive slope for feeding *versus* availability of the conspecific prey. Hence, the easier is the access to a dead conspecific, the greater will be the number of cannibals. Except for *D. virilis*, all species presented, at least for one of the prey types, a significant slope with a high R^2 (i.e. $R^2 > 0.60$) Therefore, most species had a good adherence to a positive, non-horizontal equation which suggests that readily available dead individuals are likely to be cannibalized.

Species	***Larvae with coloured gut***				
Replicate	***1***	***2***	***3***	***4***	***TOTAL*** *N (%)*
D. mediopunctata	3	5	4	3	15(37.5%)
D. immigrans	1	0	0	1	2(5%)
D. bandeirantorum	0	0	0	0	0
D. virilis	0	0	0	0	0
D. ornatifrons	1	0	0	7	8(20%)
D. melanogaster	3	5	2	6	16(40%)
D. simulans	7	7	5	5	24(60%)

Table S8. ***Data of the number on larvae that fed on chorion.*** The counts represent the number of larvae that had coloured material in their gut, which was a result of feeding on coloured eggs. As only the chorion was coloured, this indicates occurrence of cannibalism of chorion. The percentage indicates the proportion of the larvae that engaged at some point in cannibalistic behaviour.

Species									***TOTAIS***	***FISHER EXACT TEST against D^LT^L N = 60; α = 0.0167***
Replicate			***1***	***2***	***3***	***4***	***5***	***6***		***p-value***
D. mediopunctata	**TREATMENTS**	D^LT^L	0	0	0	0	0	0	0	
		D^HT^L	0	0	0	0	0	0	0	1.0000
		D^LT^H	0	4	2	0	0	0	6	**0.0024**
		D^HT^H	0	1	0	0	0	0	1	1.0000
D. melanogaster		D^LT^L	0	0	0	0	0	0	0	
		D^HT^L	3	2	1	1	1	5	13	**>0.0001**
		D^LT^H	0	2	1	4	2	4	13	**>0.0001**
D. simulans		D^LT^L	0	0	0	1	0	1	2	
		D^HT^L	0	0	1	0	1	0	2	1.0000
		D^LT^H	0	0	1	0	3	0	4	0.6707
		D^HT^H	0	2	1	2	1	0	6	0.2542

Table S9. ***Data and Statistics of the number of eggs that had embryos exposed by larval cannibalisation.*** Values in bold are statistically significant; we performed a Bonferroni correction on the α values, using m = 3. The counts represent the number of eggs on each replicate that were found with the chorion violated, therefore exposing the embryo to consumption. As the only agents in the assay were the potential larvae, this violated eggs were used as a measure of the exposure of embryos to cannibalisation. DL = 10 individuals; DH = 60 individuals; TL = 3 hours; TH = 18 hours.

Replicate	*D. melanogaster*							
	25-egg Replicate				*100-egg Replicate*			
	Adults	***Pupae***	***Fungi***	***Perforation***	***Adults***	***Pupae***	***Fungi***	***Perforation***
1	0	0	Y	Y	0	0	N	N
2	0	0	Y	Y	0	0	N	Y
3	0	0	Y	N	0	0	Y	N
4	0	0	Y	Y	1	1	N	N
5	0	0	Y	Y	0	0	N	N
6	0	0	N	N	0	0	N	Y
7	0	0	N	N	0	0	Y	Y
8	0	0	N	N	0	0	Y	N
9	0	0	N	N	0	0	Y	Y
10	0	0	N	N	0	0	N	N
11	0	0	N	N				
12	0	0	Y	N				
13	0	0	Y	Y				
14	0	0	N	N				
15	0	0	N	Y				
16	0	0	Y	Y				
17	0	0	Y	Y				
18	0	0	Y	N				
19	0	0	N	N				
20	0	0	N	N				
21	0	0	N	Y				
22	0	0	N	N				
23	0	0	Y	N				
24	0	0	Y	N				
25	0	0	Y	Y				
26	0	0	Y	Y				
27	0	0	Y	Y				
28	0	0	Y	N				
29	0	0	N	N				
30	0	0	Y	Y				
31	0	0	Y	N				
32	0	0	Y	N				
33	0	0	Y	N				
34	0	0	Y	Y				
35	0	0	N	N				
36	0	0	N	N				
37	0	0	Y	N				
38	0	0	N	N				
39	0	0	Y	N				
40	0	0	Y	N				
TOTAL	**0**	**0**	**60%**	**35%**	**1**	**1**	**40%**	**40%**

Table S10. ***Number of individuals that developed through cannibalism in D. melanogaster.*** The count represents the number of individuals that were found in the isolated dish after 30 days. As apart from agar and sugar no nutrient was provided, the difference in viability between the high-density (100 individuals per plate) and the low-density (25 individuals per plate) treatments are considered to be result of cannibalism. The observation of fungi and perforation in the agar were made using a stereo microscope.

Replicate	D. SIMULANS							
	25-egg Replicate				100-egg Replicate			
	Adults	Pupae	Fungi	Perforation	Adults	Pupae	Fungi	Perforation
1	0	0	Y	N	1	1	Y	Y
2	0	0	Y	Y	1	1	Y	Y
3	0	0	Y	Y	0	0	Y	N
4	0	0	Y	Y	0	0	Y	Y
5	0	0	Y	N	0	0	N	N
6	0	0	Y	Y	0	0	Y	Y
7	0	0	N	Y	0	0	Y	Y
8	0	0	N	Y	1	1	Y	Y
9	0	0	Y	N	1	1	Y	Y
10	0	0	Y	Y	1	2	N	Y
11	0	0	Y	Y				
12	0	0	Y	Y				
13	0	0	N	Y				
14	0	0	N	Y				
15	0	0	N	Y				
16	0	0	Y	Y				
17	0	0	Y	Y				
18	0	0	Y	Y				
19	0	0	N	Y				
20	0	0	Y	Y				
21	0	0	Y	Y				
22	0	0	Y	N				
23	0	0	Y	Y				
24	0	0	N	Y				
25	0	0	N	Y				
26	0	0	N	N				
27	0	0	N	Y				
28	0	0	N	Y				
29	0	0	N	Y				
30	0	0	Y	Y				
31	0	0	N	Y				
32	0	0	N	Y				
33	0	0	Y	Y				
34	0	0	N	Y				
35	0	0	Y	Y				
36	0	0	N	Y				
37	0	0	Y	Y				
38	0	0	N	Y				
39	0	0	Y	Y				
40	0	0	N	Y				
TOTAL	**0**	**0**	**55%**	**87.5%**	**5**	**6**	**80%**	**80%**

Table S11. ***Number of individuals that developed through cannibalism in D. simulans.*** The count represents the number of individuals that were found in the isolated dish after 30 days. As apart from agar and sugar no nutrient was provided, the difference in viability between the high-density (100 individuals per plate) and the low-density (25 individuals per plate) treatments are considered to be result of cannibalism. The observation of fungi and perforation in the agar were made using a stereo microscope.

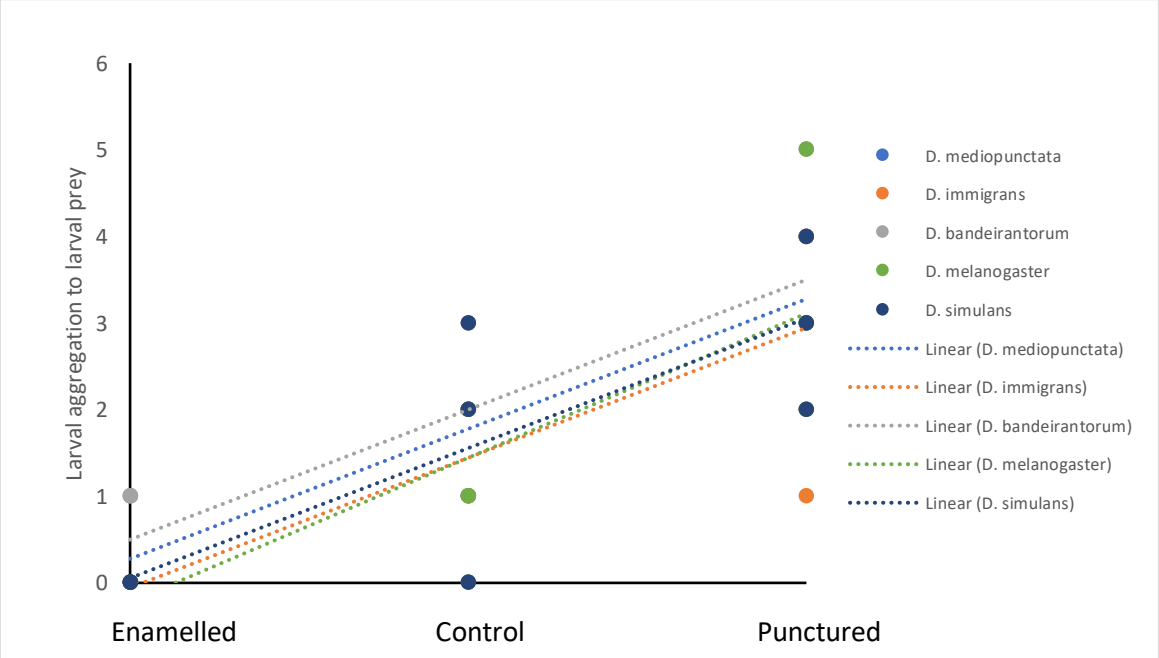
	Larval Length (mm)	
Number	Pool 3+	Pool 2-
1	0.58	0.65
2	0.60	0.73
3	0.66	0.82
4	0.68	0.87
5	0.70	0.90
6	0.74	0.91
7	0.75	0.92
8	0.76	0.92
9	0.79	0.94
10	0.80	0.95
11	0.81	0.96
12	0.82	0.96
13	0.83	0.98
14	0.84	0.98
15	0.89	0.98
16	0.91	0.99
17	0.94	0.99
18	0.96	1.00
19	0.96	1.00
20	0.97	1.04
21	0.98	1.04
22	0.98	1.04
23	0.98	1.04
24	0.99	1.04
25	0.99	1.05
26	1.00	1.05
27	1.00	1.07
28	1.02	1.07
29	1.02	1.07
30	1.02	1.07
31	1.02	1.09
32	1.02	1.10
33	1.03	1.10
34	1.04	1.10
35	1.05	1.10
36	1.05	1.11
37	1.06	1.12
38	1.07	1.14
39	1.08	1.14
40	1.08	1.15
41	1.09	1.15
42	1.09	1.15
43	1.09	1.16
44	1.09	1.16
45	1.10	1.16
46	1.10	1.16
47	1.11	1.18
48	1.12	1.18
49	1.13	1.18
50	1.13	1.20
51	1.13	1.20
52	1.13	1.20
53	1.13	1.21
54	1.14	1.22
55	1.14	1.22
56	1.14	1.22
57	1.14	1.24
58	1.14	1.27
59	1.14	1.28
60	1.14	1.29
61	1.14	1.29
62	1.15	1.31
63	1.15	1.34
64	1.15	1.38
65	1.15	1.41
66	1.15	1.44
67	1.15	1.44
68	1.15	1.57
69	1.16	1.73
70	1.16	1.81
71	1.16	
72	1.16	
73	1.16	
74	1.17	
75	1.17	
76	1.19	
77	1.19	
78	1.20	
79	1.20	
80	1.20	
81	1.22	
82	1.22	
83	1.23	
84	1.23	
85	1.23	
86	1.24	
87	1.25	
88	1.26	
89	1.27	
90	1.27	
91	1.28	
92	1.28	
93	1.28	
94	1.29	
95	1.29	
96	1.31	
97	1.32	
98	1.32	
99	1.32	
100	1.33	
101	1.34	
102	1.36	
103	1.62	
104	1.72	
105	1.82	
106	1.82	
107	1.95	
108	1.99	
109	2.93	
MEDIA	**1.1394**	**1.1276**
STDEV	**0.2957**	**0.1968**

Table S12. ***Length of preserved larvae from the strains Pool 3+ and Pool 2-.*** The length (mm) of normally fed larvae was used as an indicator of size for two strain. These strains were obtained from artificial selection for size in an environment where cannibalism would be advantageous. The measures were obtained from photos acquired using a stereo microscope and larvae preserved in a 1:1 ethanol propanol solution. The similarity in average larval size for both strains suggests that the artificial selection performed did not affect size of larvae when they are normally fed.

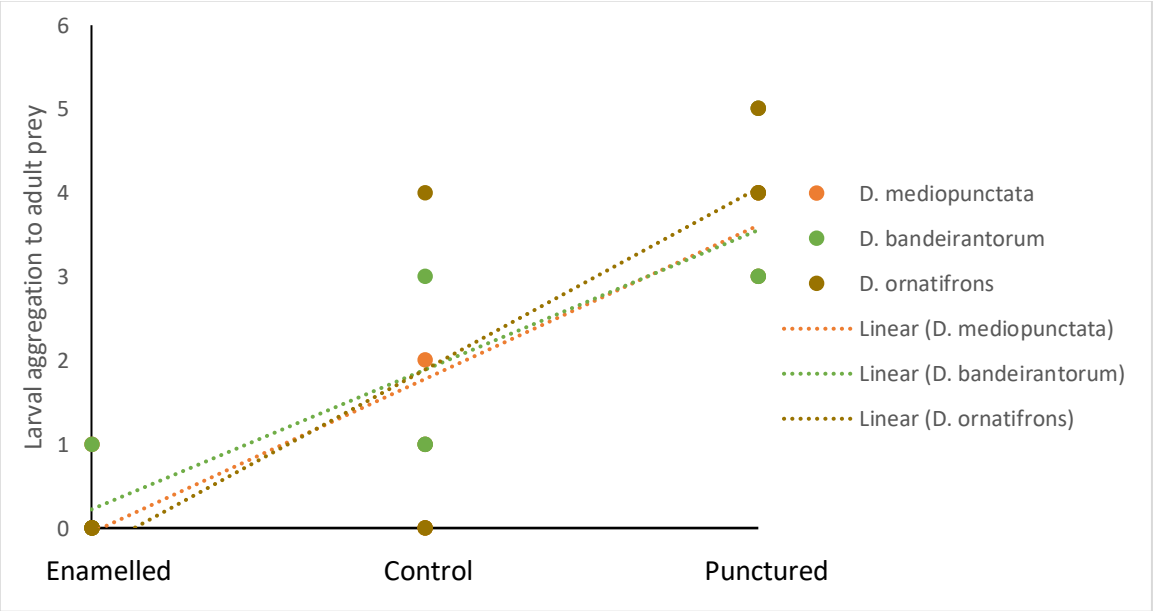
	Larval aggregation to prey	
Replicate	***3+***	***2-***
1	30	21
2	36	19
3	27	19
4	22	24
5	32	23
TOTAL	147	106
FISHER EXACT TEST α = 0.05; N = 400		
p-value	$p < 0.0001$	

Table S13. ***Data and statistics of cannibalism of strains Pool 3+ and Pool2-.*** The counts represent the number of larvae that were in direct contact with the prey, used as a parameter to evaluate cannibalism occurrence. The number of larvae cannibalising in Pool 3+ was statistically greater than in Pool 2-, suggesting that cannibalism was more frequent in Pool 3+.

3.11. SUPPLEMENTARY FIGURES



Supplementary Figure S1. Linear regressions of the aggregation of potential cannibal larvae around larval prey in three degrees of accessibility. Control is the group where prey was not enamelled nor punctured. The more accessible the prey, the greater was the number of larvae in contact with it. All species presented a positive slope, but we only plotted the regressions with a significant p-value and $R^2 > 0.6$ (F-test; Supplementary Table S7).



Supplementary Figure S2. Linear regressions of the aggregation of potential cannibal larvae around adult prey in three degrees of accessibility. Control is the group where prey was not enamelled nor punctured. The more accessible the prey, the greater was the number of larvae in contact with it. All species presented a positive slope, but we only plotted the ones with a significant p-value and $R^2 > 0.6$ (F-test; Supplementary Table S7).

4. ARTIGO 3: *Spiroplasma poulsonii* em *Drosophila melanogaster* em privação nutricional: filhas de mães infectadas são maiores, se desenvolvem mais rápido e canibalizam mais

Artigo em processo de submissão.

THE DAUGHTERS OF *SPIROPLASMA POULSONII* INFECTED FEMALES OF *DROSOPHILA MELANOGASTER* CANNIBALISE MORE, ARE LARGER IN LARVAL STAGE AND DEVELOP FASTER WHEN NUTRITIONALLY DEPRIVED

Cesar Henrique Mondini[1,*] and Louis Bernard Klaczko[1]

[1]Department of Genetics and Molecular Biology, UNICAMP, 13083 – 970, Brazil
[*]corresponding author: cesarmondini@hotmail.com; ORCID 0000-0002-4677-3493

4.1. ABSTRACT

Spiroplasma poulsonii is a reproductive parasite that provokes male killing in *Drosophila melanogaster. S. poulsonii* has imperfect vertical transmission, and theoretical work asserts that this must lead to the extinction of male-killers in populations. However, one way the infection could persist over evolutionary time would be through reallocation of resources from the dead brothers to living sisters. Here, we examined whether *S. poulsonii* infections in *D. melanogaster* enhance cannibalism, and whether, for larvae raised in poor nutritional environments, *S. poulsonii* offers any ecological benefits to the daughters of infected females, as a putative by-product of the reallocation of resources from brothers to sisters. To do so, we compared traits of *D. melanogaster* infected and uninfected with *S. poulsonii*, raised in the first 72h in medium with only agar, sugar, and water. We found that, when in very poor conditions, *S. poulsonii* enhances cannibalism in the offspring of *D. melanogaster*, that the daughters of infected mothers become larger larvae, and that they develop faster when compared to their uninfected counterparts. Our results suggest that these effects are environment-dependent, since they are in part the opposite of data available in the literature and the main difference from our studies is that our assays were held in poor environments. These effects may be especially advantageous in *Drosophila* because this group tends to have competitive larval sites. We discuss that this environment-dependent effects are especially valuable in invading non-male-killer populations and in geographical expansions. Moreover, these effects must be considered as part of an intricate scenario where horizontal transmission may be very important. Finally, we argue that these effects might be caused by the reallocation of resources from brothers to sisters in these nutritionally-deprived scenarios, but further work is needed to crystalize the cause-effect relationship between greater rates of cannibalism and adaptive life history traits.

KEYWORDS: resource reallocation, reproductive parasite, cannibalism, *Spiroplasma*.

4.2. DECLARATIONS

FUNDING: this study was funded by FAPESP, CAPES and CNPq.

CONFLICTS OF INTEREST/COMPETING INTERESTS: there are no conflicts of interests.

AVAILABILITY OF DATA AND MATERIAL: all data generated or analysed during this study are included in this published article and its supplementary information files.

CODE AVAILABILITY: does not apply.

AUTHORS' CONTRIBUTIONS: original idea: CHM and LBK; experimental design: CHM. and LBK; experimental execution: CHM; data analysis: CHM and LBK; manuscript: CHM and LBK.

ADDITIONAL DECLARATIONS FOR ARTICLES IN LIFE SCIENCE JOURNALS THAT REPORT THE RESULTS OF STUDIES INVOLVING HUMANS AND/OR ANIMALS: does not apply.

ETHICS APPROVAL: does not apply.

CONSENT TO PARTICIPATE: does not apply.

CONSENT FOR PUBLICATION: does not apply.

4.3. INTRODUCTION

It has been 60 years since Donald F. Poulson and Bungo Sakaguchi observed microscopic filaments in *Drosophila* that produced female-only progenies [1]. Today we know that those filaments were the maternally-transmitted bacteria *Spiroplasma poulsonii* [2]; that this bacterium causes male-killing in natural populations of *Drosophila melanogaster* [3]; and that this male-killing is produced by the bacterial protein *Sp*AID that targets the dosage compensation machinery of the male X chromosome, promoting apoptosis [4]. We have also learned that *Spiroplasma* is the second most common heritable symbiont in insect hosts [5]; that 16 species of *Drosophila* can be infected with *Spiroplasma* [6]; and that, in eight *Drosophila* species, *Spiroplasma* can cause male-killing [7]. Nevertheless, although much has been discovered about the host, the parasite, and the interactions between them, one question remains unanswered: how can this infection thrive and persist?

The evolutionary conundrum surrounding male-killing *S. poulsonii* in *D. melanogaster* is: – if vertical transmission of the male-killing symbiont is not perfect, infection should disappear over evolutionary time [8]. Being a male-killing bacterium with imperfect vertical transmission [3], *S. poulsonii* infections in *D. melanogaster* are expected to go extinct. Hurst and Majerus [8] theoretical work describes how such infections may remain in populations. They propose three mechanisms: (1) resource reallocation; (2) inbreeding avoidance; and (3) promotion of horizontal transmission. However, for all three mechanisms, one key concept is that the death of the males must be the factor that will, indirectly, affect the females. This means that: in (1) resource reallocation, the available resources must be transferred from males to females, either directly by cannibalisation or indirectly by reduced competition; in (2) inbreeding avoidance, the death of males must reduce inbreeding in the females' offspring; and in (3) promotion of horizontal transmission, the dead males must induce or allow horizontal transmission of the symbiont to uninfected females. Therefore, any advantageous effects that *S. poulsonii* might produce in *Drosophila* that are not dependent of male death, albeit important, will not avoid the evolutionary constraints of being a heritable male-killing symbiont with imperfect vertical transmission.

Although fitness benefits, through protection from parasitoid wasps, are produced in the lab in *D. melanogaster* infected with male-killing *S. poulsonii* [9,10], this is an instance of fitness benefit that is independent of the death of males. On the

other hand, investigations on the advantages that depend on the death of males are ambiguous. Although phylogenetic evidence indicates previous events of horizontal transmission [11] and such transmission has been replicated using living flies and mites [12], no evidence that the transfection depends on male death was presented. Moreover, so far, no investigations on the role of male-killing in avoiding inbreeding in *D. melanogaster* have been published, as far as we know. Resource reallocation, on the other hand, has some promising evidence: even though Montenegro *et al* [13] did not find any fitness benefits in *D. melanogaster* infected with *S. poulsonii*, Martins *et al* [14] work showed increased fecundity in infected females from nature, as well as slightly larger daughters from infected mothers raised in the laboratory. Advancing resource reallocation's case, it has been observed in other species. It has been detected in *Drosophila innubila* infected with another male-killer heritable symbiont, *Wolbachia* [15]; it has also been detected in ladybirds, in which *Spiroplasma* was shown to induce cannibalism [16], increase the number of produced daughters [17] and to promote greater response when infected larvae cannibalise eggs [18]. In ladybirds it is clear that resource reallocation depends on cannibalism.

Cannibalism was shown in *D. melanogaster* larvae that cannibalise other larvae [19] as well as eggs [20]. Moreover, *Drosophila* is known to develop in limited sites with crowding and competition [21, 22], and nutritional stress induces cannibalism in *D. melanogaster* [20]. As the resource reallocation hypothesis has been corroborated in *Drosophila* in a certain extent, taken together with the fact that cannibalism, the basis of direct reallocation of resources, has recently been detected in *Drosophila*, we here investigate whether cannibalism in *D. melanogaster*, when in nutritionally-poor environments, can provide adaptive traits to the daughter of infected mothers, furthering the case of the reallocation of resources from dead brothers to living sisters.

4.4. METHODS

BIOLOGICAL MATERIAL

We created a strain called Spiro+ using *D. melanogaster* (Canton-S) individuals in which we transfected *S. poulsonii,* following the protocols described in Martins *et al* [14]. Transfection of the bacteria was performed using haemolymph

injections from RED 42, a naturally infected *D. melanogaster* strain, courteously sent to us by Mariana Mateos, from Texas A&M University. These two *D. melanogaster* strains, Canton-S and Spiro+, were used to compare the effects that the bacteria caused upon the flies because both strains were virtually genetically identical.

All individuals were raised on standard cornmeal-molasses medium and kept at 23ºC on all occasions, given that *S. poulsonii* infections are known to be sensitive to low temperatures [23].

The eggs used on both essays were obtained by placing 2-week-old female adults in a bottle with a water-agar-sugar mixture (1.5% agar; 4% sugar) for 3 hours, to ensure uniformity of hatching times. These eggs were both used as potential prey, when used immediately, as well as potential cannibals, when they were allowed to develop for 3 days in a petri dish with the same water-agar-sugar mixture and nothing else to feed.

ENHANCEMENT OF CANNIBALISM BY *S. POULSONII* INFECTION ASSAY

Petri dishes with a water-agar-sugar mixture (1.5% agar; 4% sugar) were assembled using 40 3-day-old larvae as potential cannibals and 1 dead punctured adult as potential prey. Control replicas were assembled with Canton-S larvae, whereas Treatment replicas were assembled with Spiro+ larvae. Both Control and Treatment potential prey were Canton-S. Each replicate ran for 2 hours and then the number of larvae in direct contact with the punctured adult was counted and used as an estimate of cannibalism frequency, as the aggregated larvae were observed feeding on the dead conspecific.

INFECTION EFFECT ON SIZE AND DEVELOPMENT ASSAY

Set-up and nutritional availability

We placed eggs in Petri dishes with a water-agar-sugar mixture (1.5% agar; 4% sugar), using 100 eggs in the dishes with Canton-S eggs and 200 in the dishes with Spiro+ eggs, since this strain of *S. poulsonii* has a strong male-killing effect and it was previously demonstrated that flies infected with it produce no males [14]. We created environments with the same density, but in the Spiro+ dishes there are eggs

that would not hatch or die in early stages of development, and therefore could potentially be consumed by the larvae. This represents a potential difference in available nutrients between treatments, but in both cases nutritional input is compromised, given that the water-agar-sugar mixture is extremely poor in nutrients. A difference in nutrition could be obtained, for example, through cannibalization of unhatched eggs and/or larvae.

For each treatment, 10 replicas were assembled. The individuals were kept in the dishes for 72 hours.

Larval and pupal size

After 72 hours, half of the larvae of each dish, randomly chosen, was transferred to an ethanol-methanol solution (1:1), photographed and measured. To standardize larval size, we used the longest possible straight line that would fit the entire larval body.

For each dish, the remaining half of the larvae was placed in separate vials with standard cornmeal-molasses medium and monitored until emergence as imagoes. Once the pupa hatched, it was collected, photographed, and measured using the method described by Arquier *et al* [24].

Adult size and development time

Daily, at the same time, all vials were inspected for emerged adults. They were sacrificed using low temperatures, photographed, and measured. To standardize adult size, we used the longest possible straight line that would fit the lateral length of the thorax (this being the length from the tip of the scutellum to the anterior margin of the thorax). Also, the day the adult emerged was used as the measure of development time.

IMAGE ACQUISITION

All images were obtained using a Nikon® DS-Fi2 camera attached to a SMZ 1000 zoom stereo microscope, and the software NIS-Elements®.

4.5. RESULTS

S. POULSONII INFECTION INDUCES CANNIBALISM IN *D. MELANOGASTER*

Out of the 240 larvae that were placed in the Petri dishes (six replicas with 40 larvae each); 198 (82.50% ± 2.05%) of the larvae that were transfected with *S. poulsonii* were engaging in cannibalism when the assay was over, whereas 177 (73.75% ± 3.52%) of the uninfected larvae were cannibalizing at the same point (Figure 1). This difference is statistically significant (Online Resource 1; Fisher's Exact Test, $X^2 = 5.376$, $p = 0.020$). The transfection provided a scenario where the genetic makeup of individuals from both groups is virtually the same; therefore, any differences between them were necessarily driven by the symbiont. This means that *S. poulsonii* infection modulated the larval behaviour, resulting in a higher occurrence of cannibalism when in a starvation-inducing habitat.

Fig. 1 Three-day-old larvae cannibalizing on conspecific punctured adult. The eggs here were inside the female that was punctured.

S. POULSONII INFECTION IN *D. MELANOGASTER* AFFECTS SIZE AND DEVELOPMENT TIMES IN POOR AND COMPETITIVE ENVIRONMENTS

Infection makes daughters become larger larvae in crowded sites

Infection with *S. poulsonii* in competitive and nutritionally-poor conditions resulted in larger larvae than uninfected ones. The offspring of transfected females that spent the first 72 hours after oviposition in a poor environment presented bigger larvae, smaller pupae, but no difference in adult size than the uninfected offspring (Figure 2).

The larvae were larger in Spiro+ then in Canton-S, with Canton-S larva measuring, on average (± standard-error), 1.005 ± 0.016 mm, whereas Spiro+ larva measured, on average, 1.603 ± 0.032 mm, a statistically significant difference (Online Resource 2; $T = 18.21$, $df = 449$ and $p < 0.0001$). This indicates that the effect that *S. poulsonii* causes in starvation-induced *D. melanogaster* is maximal in early stages of development. This suggests that *S. poulsonii* is advantageous in very competitive larval systems.

Contrastingly, pupae transfected with *S. poulsonii* were significantly smaller than uninfected ones (Online Resources 3. $T = 4.63$, $df = 188$ and $p < 0.0001$; Canton-S 1.964 ± 0.020 mm; Spiro+ 1.860 ± 0.023 mm). Furthermore, adults presented no statistical difference in thorax size (Online resource 4. $T = 0.92$, $df = 186$ and $p = 0.3607$; Canton-S 1.015 ± 0.051 mm; Spiro+ 1.021 ± 0.037 mm). This is evidence that size effects that *S. poulsonii* infection may cause in larval stages produces minor consequences in later development stages.

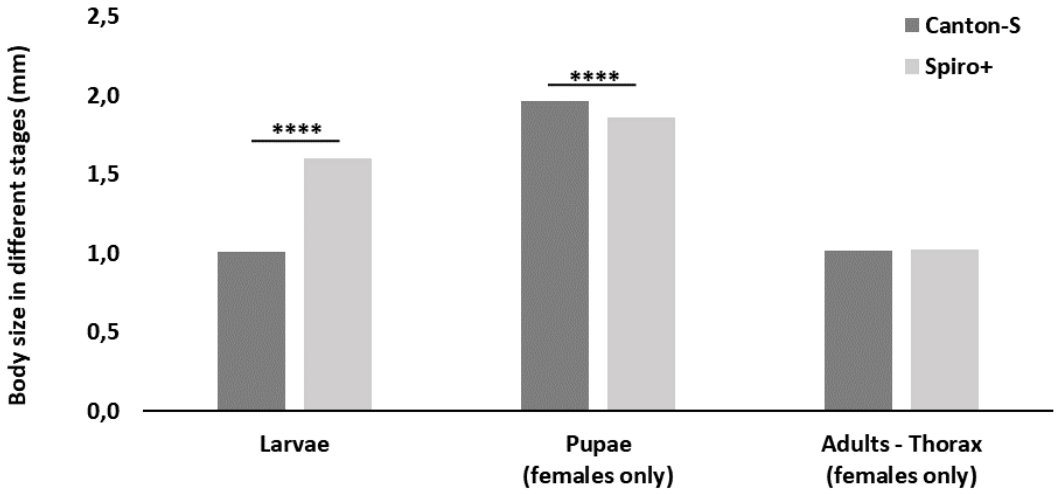


Fig. 2 Measures of body size in three development stages. Error bars are not visible because they are smaller than the image's resolution. Non-paired Student's t-test: **** represents $p < 0.0001$

Infection does not increase production nor survivability of daughters in crowded sites

Infection with *S. poulsonii* in competitive and nutritionally-poor conditions did not result in higher production nor survivability of females. Although Spiro+ replicas were assembled with 200 eggs, which was double the number of eggs used in the Canton-S replicas, for larvae, pupae and adults, the average number of individuals per replicate was lower or very similar in the transfected flies (Figure 3A). Canton-S had, on average, 61.11 ± 4.46 larvae, 17.80 ± 2.24 pupae and 14.70 ± 1.62 adults per replicate, whereas Spiro+ had, on average, 35.80 ± 3.97 larvae, 14.60 ± 1.91 pupae and 14.40 ± 1.91 adults per replicate (Online Resource 5).

It is noteworthy that as the time passed, less accentuated the difference between the number of individuals in the two treatments became, to a point where the number of final adults were virtually the same. While the difference in the number of larvae between treatments was significant (Online Resource 5; T = 5.24, df = 17 and p = 0.0005), the difference in the number of pupae and adults was not (Online Resource 5. Pupae: T = 1.09, df = 18 and p = 0.2920; Adults: T = 0.92, df = 18 and p = 0.3706). This suggests that the only survivability effect produced in the offspring, in the end, was killing males. This is made clear in Figure 3C, given that as the individuals developed, the survivability of Canton-S individuals got progressively closer to double the survivability of Spiro+, despite the survivability of Canton-S larvae being more than the triple of the survivability of Spiro+.

Regarding the male killing effect of *S. poulsonii*, only 2 out of 124 (1.6%) emerged adults were male within the transfected flies, whereas 67 out of 144 (46.5%) adults were male in the uninfected replicas. Figure 3B shows that when we correct the number of females that emerged taking into consideration the starting number of eggs (*i.e.,* doubling the number of females from the Canton-S replicas), the number of produced females did not differ between treatments (Online Resource 5; T = 0.43, df = 18 and p = 0.6741). This is evidence that male-killing *S. poulsonii* did not increase the survivability of daughters in poor environments.

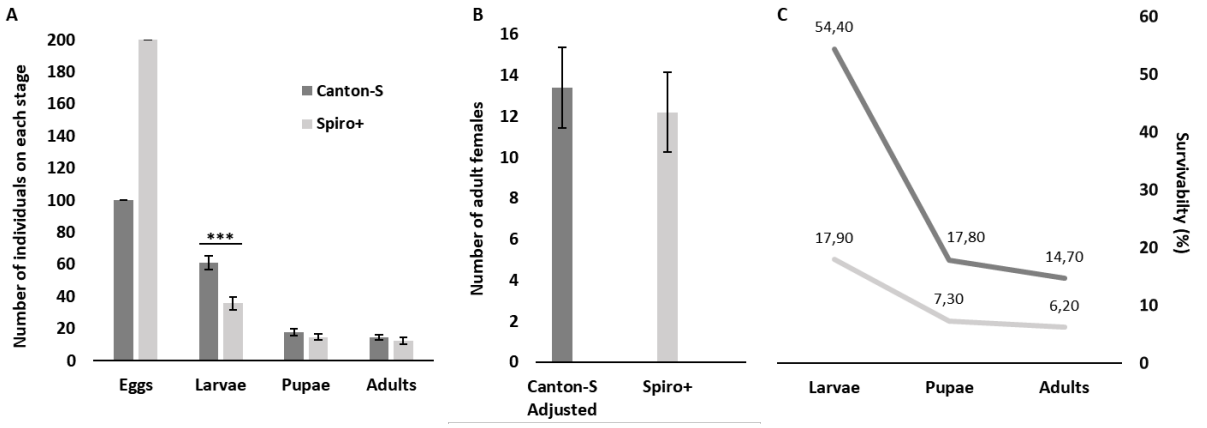


Fig. 3 Descriptive statistics of the viability of infected (Spiro+) *versus* uninfected (Canton-S) lineages. A) Average number of individuals per replicate; bars represent standard error. B) Average number of adult females for each treatment per replicate; the number of females in Canton-S was doubled to match the original number of eggs in Spiro+ replicas; bars represent standard error. C) Percentage of individuals that survived, in average, per replicate, from the total amount of eggs. Even though Spiro+ replicas started with twice as many eggs as Canton-S did, the average number of pupae and adults are very similar. This means that the viability of Spiro+ individuals are, roughly, half the survivability of Canton-S ones. Non-paired Student's t-test: *** represents $p < 0.001$.

Infection makes daughters develop faster in crowded sites

Infection with *S. poulsonii* in competitive and nutritionally-poor conditions resulted in faster development. Daughters of transfected females (Spiro+) took, on average, 13.21 ± 0.65 days to emerge as adults, whereas daughters uninfected ones (Canton-S) took, on average, 14.49 ± 0.66 days (Figure 4). This difference is statistically significant (Online Resource 6; T = 12.79, df = 186, p < 0.0001). This indicates that, when eggs of *D. melanogaster* are placed in poor resource habitats, infection with *S. poulsonii* can be adaptive, allowing then to develop faster than uninfected conspecifics.

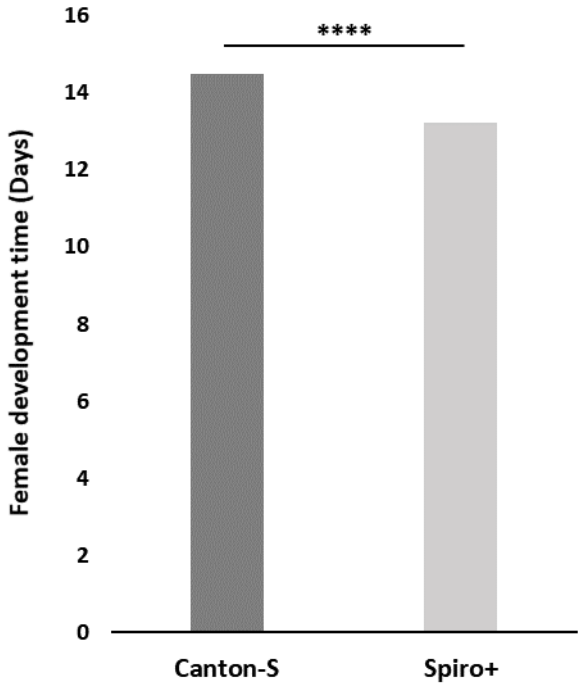


Fig. 4 Female development time from eggs to emergence as adults. The lineage infected with *S. poulsonii*, Spiro+, showed a statistically significantly shorter development time than the uninfected one, Canton-S. Error bars are not visible because they are smaller than the image's resolution. Non-paired Student's t-test: **** represents p < 0.0001

4.6. DISCUSSION

Our findings show enhancement of the cannibalistic behaviour in *D. melanogaster* promoted by male-killing *S. poulsonii*, which affects the evolutionary history of the infection. Cannibalism is widespread and impacts population structure and evolution; it is also modulated by crowding and food deprivation [25], which are the driving factors in our assays. Nakamura [16] has demonstrated that, in the ladybird *Harmonia axyridis*, male-killing *S. poulsonii* increased egg cannibalism among siblings, which reallocated resources from dead brothers to sisters. We here argue that the enhancement of cannibalism in *D. melanogaster* has potentially an equivalent importance, allowing *S. poulsonii* infections to persist in populations.

Cannibalism is a mechanism to reallocate resources in *Drosophila*. It has been experimentally selected in *D. melanogaster* [19], which demonstrates that there is a genetic basis for this behaviour. In addition, cannibalism in *D. melanogaster* was shown to be enhanced by nutritional stress and to be beneficial in such conditions [20]. Therefore, when in starvation, enhancement of cannibalism by heritable endosymbionts is possible and would be favoured by natural selection, as demonstrated in ladybirds [16,18]. This is especially important in *D. melanogaster*, which oviposits in patchy patterns, specifically in fermenting fruit, with limitations in space and time, similarly to *Drosophila willistoni* [21]. This results in development sites that are crowded and competitive. Such limitations in food intake set up a scenario where minor advantages in nutrition provide future advantages in competition. Given that the genomic analysis of male-killing *S. poulsonii* suggests dependence of the bacteria on the host [26], the enhancement of cannibalism provided by *S. poulsonii* in *D. melanogaster* in nutritionally-poor environments is advantageous for both organisms. The enhanced rate of cannibalism we observed is, therefore, leading to the reallocation of some resources from brothers to sisters, but it remains unclear whether this is evolutionary relevant, *i.e.* if the benefits from those resources are significant.

We present evidence that, in *D. melanogaster*, mothers infected with *S. poulsonii* produce larvae that are larger than those produced by uninfected mothers. This is the first investigation of the effect of such infection on larval size in literature. It is important to note that the difference in larval size was obtained from larvae that were not sexed, and the Spiro+ produces almost exclusively females [24], which are larger than males, whereas Canton-S produces males and females in an approximate 1:1

ratio. Although this should affect average larval size, various sources in literature [27, 28 and 29] shows that, at 72h, the time we took the measures, females are no more than 30% larger than males. In our data, the average Spiro+ larval size was approximately 60% larger than the average Canton-S larval size (Spiro+ = 1.603 ± 0.032; Canton-S = 1.005 ± 0.016), and a comparison of lineages where sexual dimorphism of 30% is the only driving factor would lead to a size difference of 13% between these strains. This indicates that sex alone cannot explain the size difference and there is indeed a larval size difference. It has been shown that, in the ladybird *Propylea dissecta,* the consumption of eggs led first-instar larvae to be heavier and develop faster when compared with aphid-fed larvae [30], indicating that cannibalism in early stages can be adaptive. We created a scenario where these relationship between cannibalism an larval size could be observed: the Petri dishes' medium (agar-water-sugar) was starvation-inducing; we excluded density as a confounding factor, given that we used 2-week-old females, in which male-killing takes place vastly in embryonic stages [31], which led to a similar number larvae (100), with different numbers of unviable eggs (100 for Spiro+, 0 for Canton-S).

Taking in account all our data, we can put forward a working hypothesis, that the enhancement of cannibalism by *S. poulsonii* leads to the cannibalisation of the unviable eggs and the larvae that perish from starvation before pupation, and such nutritional intake leads to larger larvae. We indeed observed a higher occurrence of cannibalism in infected larvae, and we also observed that infected females produced larger larvae when the larvae are raised in poor environments with dead conspecifics available, but we cannot unquestionably connect these events as cause and consequence because, despite the metabolic dependence that metagenomic analysis showed in *S. poulsonii* [26], there might be unknown bacterial metabolic pathways that produce the differential size of the larvae, and further studies of individual raising with differential availability of conspecifics for cannibalization are needed. Also, it has been shown that *D. melanogaster* uses pheromones to cloak the eggs and prevent cannibalism [32], which could potentially inhibit direct reallocation of resources through cannibalism. However, it has been discussed that this protection may dissipate over time [33], and the eggs we used were made available to the larvae for a long period of time, which would make these eggs unprotected. Also, the stage in which male-killing occurs depends on the age of the female [31], and therefore in natural oviposition sites some infected males should die in larval stages, then becoming available to

cannibalism regardless of any amount of protection that pheromones can provide to the egg. Nonetheless, scenarios where egg cannibalization should be the key factor for reallocation need to account for the protective layer as a strong factor, mostly right after oviposition.

The offspring of infected individuals compared to uninfected individuals gave rise to larger larvae, but there is no difference between treatments when we compare adult female size. Therefore, the effects of *S. poulsonii* in size are more relevant in earlier development stages. Previous work similarly did not find statistical differences in adult size between infected and uninfected flies both in laboratory as in natural conditions [14]. The fact that the advantage in size in larvae is not carried into adulthood suggests that any effects of *S. poulsonii* are more likely to be advantageous in larvae, which would explain why other investigations, which focused on adults, did not find other beneficial effects of male-killing infections of *S. poulsonii* in *Drosophila* [13]. The literature, in fact, points out that *Spiroplasma* in *Drosophila* produces many detrimental consequences [34]. Differently from the interaction between male-killing *Spiroplasma* and the ladybird species *Harmonia yedoensis*, which leads to larger offspring in infected females [16], our findings on survivability show that, from the same number of eggs, there is no statistical difference in the number of daughters that will emerge as adults. Thus, nearly half of the offspring, the males, dies, and there is no compensation in the number of daughters.

The hypothesis that *S. poulsonii* is advantageous to *D. melanogaster* in early development stages, disregarding environmental condition, is oversimplified. Because, although Martins *et al* [14] found evidence that daughters of infected mothers developed faster than those of uninfected ones, only 10% of the difference was estimated to be caused by male-killing *S. poulsonii*. And it is noteworthy that this difference was, approximately, 0.55 day in low density and 1.05 day in high density, showing that density impacted on development time. Haselkorn [6] suggests that ambiguous information regarding the benefits that *S. poulsonii* provides to *D. melanogaster* indicates that adaptive fitness effects may be conditional. Our results corroborate this perspective, because they suggest that male-killing *S. poulsonii* infections in *D. melanogaster* will be most advantageous to larvae when severely nutritionally-deprived. The difference in daughter development time we obtained between Canton-S and Spiro+, was, approximately, 1.28 days, and we had very reduced density effects because our replicas had similar number of larvae. The main

difference from our experiment and previous ones is that, in ours, we limited equally all larvae from acquiring nutrients. Because we measured larval size at the 72hr mark regardless of which instar larvae were at, the bigger larval size could be a by-product of the development time being shorter in Spiro+, and further studies comparing larval size in the same settings but comparing sizes of different instars independently is necessary, but for our conclusions this disentanglement is of secondary importance. That is because in the competitive oviposition sites of drosophila, being larger is beneficial for some reasons (mobility, aggression and defence) whereas developing faster is beneficial for others (avoiding further competition, leaving for less harmful environments). When we state that Spiro+ larvae are larger than Canton-S larvae we state so from the perspective that regardless of its stage, being 60% larger than your competition at a given moment is advantageous.

The discussion of the persistence of *S. poulsonii* infections in *Drosophila* must be accompanied by the discussion of horizontal transmission among species. There are field observations [35] and implications from laboratory studies [36] suggesting that male-killing infections in *Drosophila* tend to decline and disappear, whereas genomic analysis indicates reoccurrence of horizontal transmission [11]. In fact, it seems that long-term *Spiroplasma* infections may lead to selection of host's nuclear suppressors of the male-killing phenotype [37]. Therefore, we must understand the ecological and evolutionary interactions of *Spiroplasma* as complex: while horizontal transmission seems to ensure persistence in the long-term, reallocation of resources would potentially allow persistence in the short and mid-term. The reallocation of resources is a mechanism with which male-killers may invade non-male-killers' populations [37], and if the reallocation manifests in poor conditions, it represents an invading system that is most effective when populations are most vulnerable. In addition, it was shown that male-killing *Wolbachia* go to fixation in populations where siblings compete only among themselves [38]. This indicates that male-killing symbionts can become prevalent when an infected species is expanding its area of occurrence, because the distribution edges are prone to be harsher environments, and so reallocation via cannibalism would be most beneficial.

There is a body of evidence regarding *S. poulsonii* infections that must be taken together with our results: the declining prevalence of infection in natural populations [36]; the indication of recent invasions (as in the *S. poulsonii* population in Uganda derived from South America [39]); and the marks of horizontal transmission.

We presented evidence that *S. poulsonii* enhances cannibalism and provides ecological benefits; connecting these in a cause-effect link in poor environments will cast light on the reallocation of resources in this infection in *Drosophila*. When we consider populational aspects with the behavioural and ecological results here we obtain a picture of the intricate interactions and mechanisms that *S. poulsonii* possesses that avoid its extinction.

4.7. ACKNOWLEDGEMENTS

We thank KA Carvalho, MR D. Batista, FB Rocha, R Cavasini, GB Passos, IM Ventura, RES Penha and F Uno for all the input in the development of the work. We specially thank M Mateos for kindly providing *S. poulsonii*. We thank Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Fundação de Amparo à Pesquisa do Estado de São Paulo, and Conselho Nacional de Desenvolvimento Científico e Tecnológico for funding.

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4.9. ONLINE RESOURCES

ONLINE RESOURCE 1

Number of individuals engaging in cannibalism		
Replicate	**Canton-S**	**Spiro+**
1	26	36
2	26	35
3	33	32
4	30	31
5	28	32
6	34	32
Total	177	198
X^2	X^2= 5.376, p=0.020, α=0.05	

ONLINE RESOURCE 2

Larval size (mm)		
Sample	**Canton-S**	**Spiro+**
1	0.51	0.75
2	0.56	0.76
3	0.57	0.91
4	0.6	0.96
5	0.61	0.98
6	0.62	1.05
7	0.62	1.05
8	0.63	1.05
9	0.64	1.07
10	0.64	1.07
11	0.64	1.09
12	0.65	1.09
13	0.66	1.11
14	0.66	1.13
15	0.67	1.15
16	0.67	1.15
17	0.68	1.16
18	0.68	1.17
19	0.68	1.17
20	0.68	1.19
21	0.68	1.21
22	0.69	1.21
23	0.69	1.22
24	0.69	1.22
25	0.7	1.23
26	0.7	1.23
27	0.71	1.24
28	0.72	1.24
29	0.72	1.24
30	0.72	1.24
31	0.72	1.25
32	0.72	1.26
33	0.72	1.26
34	0.72	1.26
35	0.72	1.27
36	0.73	1.27
37	0.73	1.27
38	0.74	1.28
Larval size (mm)		

Sample	Canton-S	Spiro+
39	0.74	1.28
40	0.74	1.29
41	0.74	1.29
42	0.75	1.29
43	0.75	1.3
44	0.75	1.3
45	0.75	1.3
46	0.76	1.3
47	0.76	1.31
48	0.76	1.32
49	0.77	1.32
50	0.77	1.32
51	0.77	1.33
52	0.77	1.33
53	0.77	1.34
54	0.78	1.34
55	0.78	1.34
56	0.78	1.34
57	0.79	1.35
58	0.8	1.35
59	0.8	1.35
60	0.8	1.37
61	0.8	1.37
62	0.8	1.37
63	0.81	1.39
64	0.81	1.39
65	0.81	1.39
66	0.81	1.39
67	0.81	1.39
68	0.81	1.41
69	0.81	1.41
70	0.82	1.41
71	0.83	1.42
72	0.83	1.43
73	0.83	1.43
74	0.83	1.43
75	0.84	1.43
76	0.84	1.44
77	0.84	1.44
Larval size (mm)		

Sample	Canton-S	Spiro+
78	0.85	1.44
79	0.86	1.45
80	0.86	1.45
81	0.86	1.45
82	0.86	1.46
83	0.87	1.47
84	0.87	1.48
85	0.87	1.48
86	0.87	1.48
87	0.88	1.49
88	0.88	1.49
89	0.88	1.49
90	0.88	1.49
91	0.89	1.5
92	0.89	1.52
93	0.89	1.52
94	0.89	1.52
95	0.89	1.54
96	0.9	1.54
97	0.9	1.55
98	0.9	1.55
99	0.9	1.56
100	0.9	1.57
101	0.91	1.57
102	0.91	1.58
103	0.92	1.58
104	0.92	1.58
105	0.92	1.58
106	0.93	1.58
107	0.93	1.58
108	0.93	1.59
109	0.93	1.6
110	0.93	1.6
111	0.94	1.61
112	0.94	1.62
113	0.94	1.62
114	0.94	1.62
115	0.94	1.62
116	0.94	1.63
Larval size (mm)		

Sample	Canton-S	Spiro+
117	0.95	1.65
118	0.95	1.66
119	0.95	1.66
120	0.96	1.67
121	0.96	1.67
122	0.96	1.68
123	0.96	1.69
124	0.96	1.69
125	0.97	1.7
126	0.97	1.7
127	0.97	1.71
128	0.97	1.73
129	0.97	1.73
130	0.97	1.74
131	0.97	1.76
132	0.97	1.76
133	0.97	1.76
134	0.98	1.77
135	0.98	1.77
136	0.99	1.79
137	0.99	1.79
138	0.99	1.79
139	0.99	1.8
140	1	1.8
141	1	1.81
142	1	1.82
143	1	1.83
144	1	1.86
145	1	1.89
146	1.01	1.96
147	1.01	1.97
148	1.01	1.98
149	1.01	1.98
150	1.01	1.99
151	1.02	2
152	1.02	2.06
153	1.02	2.09
154	1.02	2.09
155	1.03	2.11
156	1.03	2.17
157	1.03	2.24
158	1.03	2.26
159	1.03	2.29
Larval size (mm)		

Sample	Canton-S	Spiro+
160	1.04	2.32
161	1.04	2.34
162	1.04	2.35
163	1.04	2.37
164	1.04	2.39
165	1.05	2.39
166	1.05	2.4
167	1.05	2.47
168	1.06	2.47
169	1.06	2.49
170	1.06	2.52
171	1.06	2.55
172	1.06	2.55
173	1.06	2.56
174	1.06	2.56
175	1.07	2.6
176	1.07	2.64
177	1.07	2.65
178	1.07	2.66
179	1.07	3.22
180	1.07	
181	1.07	
182	1.08	
183	1.08	
184	1.08	
185	1.08	
186	1.08	
187	1.08	
188	1.09	
189	1.09	
190	1.09	
191	1.09	
192	1.09	
193	1.11	
194	1.11	
195	1.11	
196	1.12	
197	1.12	
198	1.13	
199	1.13	
200	1.13	
201	1.13	
202	1.14	
Larval size (mm)		

Sample	Canton-S	Spiro+
203	1.14	
204	1.14	
205	1.14	
206	1.14	
207	1.15	
208	1.15	
209	1.15	
210	1.16	
211	1.16	
212	1.16	
213	1.16	
214	1.17	
215	1.17	
216	1.17	
217	1.18	
218	1.18	
219	1.18	
220	1.18	
221	1.19	
222	1.19	
223	1.21	
224	1.21	
225	1.21	
226	1.21	
227	1.22	
228	1.22	
229	1.22	
230	1.22	
231	1.22	
232	1.23	
233	1.23	
234	1.24	
235	1.24	
236	1.24	
237	1.24	
238	1.24	
239	1.25	
240	1.25	
241	1.26	
242	1.27	
243	1.27	
244	1.27	
245	1.27	
Larval size (mm)		

Sample	Canton-S	Spiro+
246	1.28	
247	1.28	
248	1.28	
249	1.29	
250	1.29	
251	1.29	
252	1.29	
253	1.3	
254	1.31	
255	1.31	
256	1.31	
257	1.33	

Larval size (mm)		
Sample	Canton-S	Spiro+
258	1.34	
259	1.34	
260	1.35	
261	1.39	
262	1.41	
263	1.55	
264	1.63	
265	1.63	
266	1.72	
267	1.82	
268	1.82	
269	2.08	

Larval size (mm)		
Sample	Canton-S	Spiro+
270	2.15	
271	2.15	
272	2.37	
N	272	179
Average	1.005	1.603
Std Dev	0.2644	0.4322
Std Error	0.0160	0.0323
T-Test	T=18.21, df=449 α=0.05, p>0.0001	

ONLINE RESOURCE 3

Pupal size (mm) - Females Only						
Sample	Canton-S			Spiro+		
	D	L	Size	D	L	Size
1	0,97	2,90	1,43	0,92	2,86	1,27
2	0,97	2,92	1,44	0,94	2,87	1,33
3	0,99	3,14	1,61	0,96	3,16	1,52
4	0,98	3,30	1,66	1,00	3,03	1,59
5	1,05	2,91	1,68	1,00	3,06	1,60
6	0,99	3,32	1,70	1,00	3,10	1,62
7	1,04	3,09	1,75	1,00	3,11	1,63
8	1,02	3,25	1,77	1,02	3,00	1,63
9	1,05	3,14	1,81	1,04	2,98	1,69
10	1,06	3,12	1,84	1,04	2,98	1,69
11	1,05	3,20	1,85	1,03	3,04	1,69
12	1,06	3,16	1,86	1,02	3,11	1,69
13	1,06	3,16	1,86	1,02	3,12	1,70
14	1,08	3,05	1,86	1,02	3,12	1,70
15	1,05	3,23	1,86	1,04	3,03	1,72
16	1,08	3,06	1,87	1,02	3,16	1,72
17	1,07	3,12	1,87	1,04	3,04	1,72
18	1,07	3,16	1,89	1,01	3,23	1,73
19	1,06	3,23	1,90	1,06	2,96	1,74
20	1,06	3,23	1,90	1,04	3,08	1,74
21	1,06	3,23	1,90	1,04	3,10	1,76
22	1,07	3,17	1,90	1,05	3,05	1,76
23	1,09	3,07	1,91	1,03	3,17	1,76
24	1,05	3,32	1,92	1,03	3,17	1,76
25	1,07	3,22	1,93	1,05	3,06	1,77
26	1,07	3,22	1,93	1,06	3,01	1,77
27	1,07	3,22	1,93	1,05	3,07	1,77
28	1,07	3,25	1,95	1,02	3,26	1,78
29	1,06	3,33	1,96	1,04	3,14	1,78
30	1,06	3,33	1,96	1,05	3,09	1,78
31	1,08	3,22	1,97	1,06	3,04	1,79
32	1,09	3,18	1,98	1,06	3,04	1,79
33	1,08	3,25	1,98	1,06	3,06	1,80
34	1,08	3,25	1,98	1,05	3,12	1,80
35	1,07	3,32	1,99	1,08	2,95	1,80
36	1,10	3,15	2,00	1,06	3,08	1,81
37	1,10	3,16	2,00	1,06	3,08	1,81
38	1,09	3,24	2,02	1,07	3,03	1,82
39	1,10	3,19	2,02	1,03	3,27	1,82
40	1,07	3,39	2,03	1,03	3,27	1,82
41	1,10	3,21	2,03	1,04	3,21	1,82
42	1,09	3,27	2,03	1,04	3,21	1,82
43	1,09	3,28	2,04	1,05	3,15	1,82
44	1,07	3,42	2,05	1,05	3,15	1,82
45	1,09	3,32	2,07	1,05	3,16	1,82
46	1,12	3,16	2,08	1,05	3,16	1,82
47	1,11	3,22	2,08	1,06	3,11	1,83
48	1,09	3,34	2,08	1,05	3,17	1,83
49	1,12	3,17	2,08	1,06	3,12	1,84
50	1,14	3,06	2,08	1,07	3,08	1,85
51	1,11	3,23	2,08	1,05	3,20	1,85

52	1,11	3,23	2,08	1,05	3,21	1,85
53	1,09	3,35	2,08	1,06	3,15	1,85
54	1,09	3,35	2,08	1,06	3,15	1,85
55	1,10	3,29	2,08	1,07	3,10	1,86
56	1,12	3,18	2,09	1,05	3,22	1,86
57	1,11	3,24	2,09	1,05	3,22	1,86
58	1,11	3,26	2,10	1,06	3,16	1,86
59	1,11	3,26	2,10	1,07	3,11	1,86
60	1,10	3,34	2,12	1,07	3,11	1,86
61	1,10	3,35	2,12	1,06	3,17	1,86
62	1,11	3,29	2,12	1,06	3,18	1,87
63	1,12	3,24	2,13	1,04	3,31	1,87
64	1,11	3,30	2,13	1,10	2,96	1,88
65	1,11	3,36	2,17	1,07	3,13	1,88
66	1,11	3,38	2,18	1,08	3,08	1,88
67	1,13	3,27	2,19	1,08	3,08	1,88
68	1,16	3,27	2,30	1,05	3,26	1,88
69				1,06	3,20	1,88
70				1,06	3,20	1,88
71				1,05	3,27	1,89
72				1,09	3,04	1,89
73				1,07	3,16	1,89
74				1,06	3,22	1,89
75				1,06	3,23	1,90
76				1,06	3,23	1,90
77				1,07	3,17	1,90
78				1,07	3,17	1,90
79				1,08	3,12	1,91
80				1,08	3,12	1,91
81				1,06	3,24	1,91
82				1,08	3,13	1,91
83				1,07	3,19	1,91
84				1,08	3,14	1,92
85				1,07	3,21	1,92
86				1,08	3,16	1,93
87				1,08	3,16	1,93
88				1,09	3,12	1,94
89				1,06	3,30	1,94
90				1,06	3,30	1,94
91				1,08	3,18	1,94
92				1,08	3,18	1,94
93				1,10	3,07	1,95
94				1,07	3,26	1,95
95				1,10	3,09	1,96
96				1,09	3,16	1,97
97				1,11	3,06	1,97
98				1,06	3,36	1,98
99				1,09	3,18	1,98
100				1,10	3,13	1,98
101				1,11	3,08	1,99
102				1,10	3,14	1,99
103				1,08	3,26	1,99
104				1,09	3,21	2,00
105				1,09	3,23	2,01
106				1,09	3,23	2,01
107				1,11	3,12	2,01
108				1,10	3,19	2,02
109				1,10	3,19	2,02

110		1,11	3,14	2,03
111		1,12	3,10	2,04
112		1,12	3,10	2,04
113		1,12	3,11	2,04
114		1,10	3,23	2,05
115		1,11	3,18	2,05
116		1,12	3,14	2,06
117		1,09	3,32	2,07
118		1,12	3,15	2,07
119		1,11	3,21	2,07
120		1,11	3,26	2,10
121		1,13	3,17	2,12
122		1,15	3,13	2,17

N	68	122
Average	1,964	1,859
Std Dev	0,1635	0,1406
Std Error	0,0198	0,0127
T-Test	T=4.63, df=188 α=0.05, p<0.0001	

ONLINE RESOURCE 4

Adult size - thorax (mm)		
Sample	**Canton-S**	**Spiro+**
1	0.84	0.91
2	0.92	0.92
3	0.92	0.95
4	0.93	0.95
5	0.93	0.95
6	0.94	0.96
7	0.95	0.96
8	0.95	0.96
9	0.96	0.97
10	0.97	0.97
11	0.97	0.97
12	0.98	0.97
13	0.98	0.97
14	0.98	0.97
15	0.98	0.97
16	0.99	0.98
17	0.99	0.98
18	0.99	0.98
19	1.00	0.98
20	1.00	0.98
21	1.00	0.99
22	1.00	0.99
23	1.00	0.99
24	1.01	0.99
25	1.01	0.99
26	1.01	0.99
27	1.01	0.99
28	1.01	1.00
29	1.01	1.00
30	1.01	1.00
31	1.01	1.00
32	1.01	1.00
33	1.02	1.00
34	1.02	1.00
35	1.02	1.00
36	1.02	1.00
37	1.02	1.00
38	1.03	1.00
39	1.03	1.01
40	1.03	1.01
41	1.03	1.01
42	1.03	1.01
43	1.03	1.01

Adult size - thorax (mm)		
Sample	**Canton-S**	**Spiro+**
44	1.03	1.01
45	1.03	1.01
46	1.04	1.01
47	1.04	1.01
48	1.04	1.01
49	1.04	1.01
50	1.04	1.01
51	1.04	1.01
52	1.04	1.02
53	1.04	1.02
54	1.04	1.02
55	1.04	1.02
56	1.04	1.02
57	1.04	1.02
58	1.05	1.02
59	1.06	1.02
60	1.08	1.02
61	1.08	1.02
62	1.08	1.02
63	1.08	1.02
64	1.08	1.02
65	1.09	1.03
66	1.10	1.03
67	1.20	1.03
68		1.03
69		1.03
70		1.03
71		1.03
72		1.03
73		1.03
74		1.03
75		1.03
76		1.03
77		1.04
78		1.04
79		1.04
80		1.04
81		1.04
82		1.04
83		1.04
84		1.04
85		1.04
86		1.04

Adult size - thorax (mm)		
Sample	**Canton-S**	**Spiro+**
87		1.04
88		1.04
89		1.04
90		1.04
91		1.04
92		1.04
93		1.05
94		1.05
95		1.05
96		1.05
97		1.05
98		1.05
99		1.05
100		1.05
101		1.05
102		1.06
103		1.06
104		1.06
105		1.06
106		1.06
107		1.06
108		1.06
109		1.06
110		1.06
111		1.06
112		1.07
113		1.07
114		1.07
115		1.08
116		1.08
117		1.08
118		1.09
119		1.09
120		1.11
121		1.13
N	67	121
Average	1.015	1.021
Std Dev	0.0512	0.0371
Std Error	0.0063	0.0034
T-Test	T=0.92, df=186 α=0.05, p = 0.3607	

ONLINE RESOURCE 5

GLOBAL NUMBER OF INDIVIDUALS					
Eggs	**Larvae**	**Pupae**	**Adults**	**Females**	
1000	544	178	147	67	Half of the larvae were removed to be sized
2000	358	146	124	122	

GLOBAL SURVIVABILITY (%)				The survivability in comparison to the initial number of individuals (eggs)	Given that half of the larvae were removed, the values for pupae and adults need to be further corrected by a factor of 2 to provide an accurate estimate of survivability
Eggs	**Larvae**	**Pupae**	**Adults**		
	54.40	17.80	14.70		
	17.90	7.30	6.20		

RELATIVE SURVIVABILITY (%)				The survivability in comparison to the previous development stage
Eggs	**Larvae**	**Pupae**	**Adults**	
	54.40	65.44	82.58	
	17.90	81.56	84.93	

NUMBER OF LARVAE PER REPLICATE			
Canton-S	**Spiro+**		
54	50		
36	18		
80	10		
62	40		
66	42		
60	32		
62	40		
52	46		
78	42		
	38		
61.11	35.80	**T test**	T=5.24, df=17 α=0.05, p=0.0005
4.46	3.97		

NUMBER OF PUPAE PER REPLICATE			
Canton-S	**Spiro+**		
11	21		
7	6		
14	18		
17	14		
19	10		
14	14		
26	27		
17	11		
22	11		
31	14		
17.80	14.60	**T test**	T=1.09, df=18 α=0.05, p=0.2920
2.24	1.91		

NUMBER OF ADULT PER REPLICATE			
Canton-S	**Spiro+**		
11	19		
7	5		
14	16		
14	11		
15	10		
11	12		
19	25		
14	8		
16	11		
26	7		
14.70	12.40	**T test**	T=0.92, df=18 α=0.025, p=0.3706
1.62	1.91		

	NUMBER OF FEMALES PER REPLICATE				
Replicate	**Canton-S**	**Canton-S Adjusted**	**Spiro+**		
1	6	12	19		
2	2	4	5		
3	8	16	16		
4	7	14	11		
5	8	16	9		
6	6	12	12		
7	9	18	25		
8	3	6	7		
9	5	10	11		
10	13	26	7		
Average	6.70	13.40	12.20	**T test**	T=0.43, df=18 α=0.025, p=0.6741
Std Error	0.99	1.98	1.95		

ONLINE RESOURCE 6

Development time (days)		
Sample	Canton-S	Spiro+
1	13	12
2	13	12
3	13	12
4	13	12
5	14	12
6	14	12
7	14	12
8	14	12
9	14	12
10	14	13
11	14	13
12	14	13
13	14	13
14	15	13
15	15	13
16	15	13
17	14	13
18	14	13
19	14	13
20	14	13
21	14	13
22	14	13
23	14	13
24	14	13
25	14	13
26	14	13
27	14	13
28	14	13
29	14	13
30	14	13
31	14	13
32	14	13
33	14	13
34	14	13
35	14	13
36	15	13
37	15	13
38	15	13
39	15	13
40	15	13
41	15	13
42	15	13
43	15	13
44	15	13

Development time (days)		
Sample	Canton-S	Spiro+
45	15	13
46	15	13
47	15	13
48	15	13
49	15	13
50	15	13
51	15	13
52	15	13
53	15	13
54	15	13
55	15	13
56	15	13
57	15	13
58	15	13
59	15	13
60	15	13
61	15	13
62	15	13
63	15	13
64	15	13
65	15	13
66	16	13
67	16	13
68		13
69		13
70		13
71		13
72		13
73		13
74		13
75		13
76		13
77		13
78		13
79		13
80		13
81		13
82		13
83		13
84		13
85		13
86		13
87		13
88		13

Development time (days)		
Sample	Canton-S	Spiro+
89		13
90		13
91		13
92		13
93		13
94		13
95		14
96		14
97		14
98		14
99		14
100		14
101		14
102		14
103		14
104		14
105		14
106		14
107		14
108		14
109		14
110		14
111		14
112		14
113		14
114		14
115		14
116		15
117		15
118		15
119		15
120		15
121		15
122		15
N	67	122
Average	14.493	13.213
Std Dev	0.6600	0.6584
Std Error	0.0806	0.0596
T-Test	T=12.79, df=187 α=0.05, p>0.0001	

5. DISCUSSÃO GERAL

Nossa estimativa de prevalência da infecção de *S. poulsonii* em *D. melanogaster* na Região Metropolitana de Campinas não obteve nenhuma mosca infectada. Este resultado é relevante pois esta é a estimativa produzida com a maior coleta já feita: nominalmente são 251 linhagens desenvolvidas, cada uma, de uma única fêmea capturada. Com tais dados, nossa investigação, através da estimativa de intervalo de confiança (Brown *et al.,* 2001), produziu um intervalo de prevalência de infecção entre 0 e 1,5%, bastante inferior aos intervalos que a literatura nos oferece (Ventura *et al*., 2012), que variam entre 0 e 17%. Nossos valores são ainda menores do que a prevalência de *Spiroplasma* em insetos em geral, que é, segundo Duron *et al.* (2008), na maior parte dos casos, entre 20 e 80%. Dado que a infecção foi encontrada na cidade de Campinas no passado (Montenegro *et al.,* 2000; Montenegro *et at*., 2005), nossos dados sugerem uma baixa prevalência da infecção, senão sua extinção completa. Tal cenário abre um leque de possibilidades sobre o comportamento da infecção de *S. poulsonii* em populações naturais de *Drosophila*, e uma reavaliação das prevalências em todos os sítios já analisados no passado seria de grande valia para entender se essa potencial extinção é fenômeno comum da infecção.

Embora prevalências baixas possam ser esperadas pela sensibilidade da infecção a frio e calor (Montenegro & Klaczko, 2004; Anbutsu *et al*., 2008) e pelos efeitos prejudiciais que podem estar associados às infecções de *Spiroplasma* em *Drosophila* (Ebbert M., 1991), não há na literatura investigação periódica de longo prazo na mesma localidade avaliando se tais infecções são flutuantes, cíclicas ou estáveis. É importante ponderar que embora normalmente se trate a história evolutiva de infecções, suas epidemias e pandemias dentro de uma perspectiva de continuidade, algumas infecções muito provavelmente, local ou globalmente, estão fadadas à extinção, e este pode ter sido o caso de Campinas. Dado que *Spiroplasma* em *D. melanogaster* gera proteção ao parasitismo (Xie *et al*., 2014), que *male-killers* podem invadir populações com infecções não *male-killers* (Zug & Hammerstein, 2018) e que há indícios de transmissões horizontais nas filogenias de *Spiroplasma* (Haselkorn *et al*., 2009), pode-se supor que as relações que regem a prevalência da infecção ao longo do tempo são complexas. Um fator adicional que pode afetar tal intrincada dinâmica é a realocação direta de recursos via canibalismo que poderia

acontecer como produto do efeito male-killer, mas para tanto é necessário que canibalismo seja compreendido com clareza em *Drosophila*.

Quanto ao canibalismo em *Drosophila*, nossos resultados demonstram o quão comum este comportamento é. Nas sete espécies testadas, que são filogeneticamente dispersas dentro do gênero, nós tivemos observação direta de alguma forma de canibalismo, e exceto por *D. virilis* e *D. bandeirantorum*, que não canibalizaram ovos, todas as espécies canibalizaram ovos, larvas e adultos. Sabe-se que as larvas de *D. melanogaster* são vorazes e se alimentam, quando em estresse alimentar, de uma grande variedade de organismos, dentre estes, indivíduos conspecíficos (Vijendravarma *et al*., 2013). Nossos dados corroboram tal perspectiva, embora haja uma pequena limitação quanto a ovos. Sabe-se que *D. melanogaster* protege seus ovos de canibalismo através de feromônios (Narasimha *et al*., 2019), mas tal proteção provavelmente se enfraquece com o tempo (Khodaei & Long, 2020). Nossos resultados indicam que de fato a camada de feromônios protetivos deixa de ser efetiva após algumas horas, e isso tornaria os ovos acessíveis ao canibalismo por tempo suficiente para que irmãs canibalizem irmãos, dado que larvas recém eclodidas podem sobreviver por dias antes de morrer por falta de alimento.

Nossos resultados apontam ainda para a importância de canibalismo de diversas formas como adaptativo, posto que observamos indivíduos chegando ao estágio adulto unicamente via canibalismo competitivo. Diferentemente de trabalhos anteriores (Vijendravarma *et al*., 2013; Gregg *et al*., 1990), em que os indivíduos conspecíficos eram fornecidos mortos às larvas canibais, no nosso experimento os indivíduos tiveram de competir e canibalizar uns com os outros ao longo de todo seu desenvolvimento, pois não havia outra fonte nutricional. Nosso resultado é uma exemplificação extrema dos benefícios que canibalismo pode oferecer em ambientes competitivos e com alta densidade populacional (Nishimura & Isoda, 2004; Getto *et al*., 2005). Considerando que locais de oviposição em *Drosophila* tendem a, justamente, ser competitivos e com alta densidade populacional (Birch & Bataglia, 1957; Atkinson W., 1979), canibalismo parece ser um importante mecanismo de realocação de recursos em *Drosophila* quando em carência de recursos, e a realocação de recursos de irmãos mortos para irmãs, via canibalismo, é um dos mecanismos propostos por Hurst & Majerus (1993) para permitir que *male-killers* perseverem no tempo evolutivo.

Obtivemos, através de seleção artificial, linhagens que engajavam em canibalismo em graus distintos. Embora possa-se admitir, inicialmente, que a seleção tenha sido de voracidade como um todo, não apenas canibalismo, tal hipótese é descartada pelo fato de que o tamanho médio das larvas de ambas as linhagens não era estatisticamente distinto. Sabe-se que o gene *for* e a via metabólica *PI3K/Atk* afetam o padrão de alimentação como um todo, mas não há evidência de relação entre estes e a via de canibalismo em *Drosophila* (Ahmad *et al.* 2018). Nossos resultados apontam, na verdade, para independência entre tais vias. Nós conseguirmos selecionar comportamento canibal (que foi medido indiretamente via agregação das larvas canibais à presa) através do tamanho das larvas, mas o tamanho das larvas não foi selecionado conjuntamente, o que seria esperado caso canibalismo fosse apenas uma manifestação de voracidade em geral.

O fato de, em *Drosophila*, canibalismo ser comum, adaptativo, sujeito a seleção natural, e se manifestar em larvas predando todos os outros estágios de desenvolvimento da mosca torna canibalismo um candidato bastante promissor para a hipótese de realocação de recursos de irmãos mortos por *S. poulsonii* para as suas irmãs. Semelhantemente ao que acontece com *Harmonia axyridis* infectada com *S. poulsonii* (Nakamura *et al.* 2006), nós detectamos que a prole de uma mãe infectada canibaliza com mais frequência. A indução comportamental de canibalismo em nosso trabalho foi detectada utilizando adultos como presas, mas não há indícios para crer que também não se manifeste quando as presas potenciais são ovos, ou ainda larvas que pereceram antes do estágio de pupa. Temos evidências que esta indução de canibalismo em *Drosophila* por *S. poulsonii* existe, mas para mostrar-se mecanismo relevante na hipótese de realocação de recursos, precisa expressar-se na utilização dos irmãos mortos como alimento, através de benefícios ecológicos claros.

Detectamos que, em *D. melanogaster,* as larvas de filhas de mães infectadas com *S. poulsonii*, quando comparadas com as filhas de mães não infectadas, são maiores no estágio larval, porém menores no estágio de pupa, e de mesmo tamanho no estágio adulto. Estudos prévios (Montenegro *et al.* 2006; Martins *et al.* 2010) também não encontraram diferença no tamanho das filhas de mães infectadas e não infectadas, mas não investigaram o impacto da infecção no estágio larval, restringindo-se ao estágio adulto. A diferença de tamanho larval é especialmente importante em locais onde há alta competição e escassez de recursos,

e nossas condições experimentais levavam esses fatores a níveis extremos. É um fenômeno que se encaixa no que se chama de mecanismo "*lifeboat*", ou salva-vidas (Getto *et al.,* 2005): poder canibalizar em situações extremas é adaptativo pois permite que o organismo atravesse os momentos mais difíceis de seu desenvolvimento.

Além de maiores, as filhas de mães infectadas também se desenvolveram mais rapidamente. Embora Martins *et al.* (2010) tenha obtido indícios de tal efeito da infecção, o fator de densidade de indivíduos parecia explicar mais amplamente a diferença em tempo de desenvolvimento do que a infecção de *S. poulsonii*. Neste trabalho, de maneira distinta, excluiu-se o fator de densidade larval ao produzir réplicas com número de larvas muito semelhantes nos diferentes tratamentos. Ao gerar placas com mesmo número de larvas, mas com número distinto de ovos (os ovos dos machos de mães infectadas com *S. poulsonii* em quase sua totalidade não deveriam ser viáveis), proporcionou-se uma configuração em que vantagens típicas, como tamanho, fecundidade, tempo de desenvolvimento e etc., seriam independentes de densidade e fruto exclusivo da infecção por *S. poulsonii*. Ao obtermos, de mães infectadas com *S. poulsonii*, larvas maiores, bem como filhas que se desenvolveram mais rapidamente, temos uma manifestação de benefício ecológico inequivocamente resultante da infecção. Uma explicação muito tentadora, e bastante plausível, é a de que os nutrientes dos irmãos são obtidos pelas irmãs canibais, e isso se manifesta fenotipicamente, fruto da realocação direta de recursos que teria potencialmente sido induzida pela maior taxa de canibalismo que a infecção por *S. poulsonii* proporcionou.

Porém não podemos afirmar que aqui temos um caso claro de realocação direta de recursos via canibalismo. De fato, como há uma maior taxa de canibalismo é evidente que há, em algum nível, realocação de recursos dos irmãos mortos para as irmãs. Porém a hipótese de realocação de recursos quanto a persistência de agentes *male-killers* proposta por Hurst & Majerus (1993) trata de uma transferência de recursos em grau elevado o suficiente para que benefícios em *fitness* sejam observados. Embora benefícios ecológicos (*i.e.* tamanho larval e tempo de desenvolvimento) tenham sido detectados, nossos dados não oferecem uma relação causa-efeito entre canibalismo e tais benefícios, de modo que se torna necessário investigar como o canibalismo afeta esses traços em indivíduos infectados quando diferentes quantidades de presa são oferecidas. Embora a análise metagenômica de *S. poulsonii* indique poucas vias metabólicas e, portanto, a dependência deste quanto

ao metabolismo do hospedeiro (Paredes *et al.,* 2015), não existe segurança completa para se descartar a possibilidade de que em privação nutricional extrema a infecção seja capaz de oferecer algum mecanismo nutricional valioso para a mosca e este seja o responsável pelos benefícios em *fitness* observados.

Ademais, os potenciais benefícios em *fitness* que a realocação de recursos via canibalismo proporcionaria para *S. poulsonii* em *Drosophila* possui um porém: nossos resultados foram obtidos dentro de uma configuração de extrema privação nutricional. Como no estudo com joaninhas que apontou que o consumo de um único ovo gera benefícios em *fitness* (Pervez *et al.,* 2006), o consumo de um único ovo, em média (possuíamos cerca de 100 larvas e 100 ovos inviáveis nas réplicas com prole de mães infectadas com *S. poulsonii*), propiciou ganhos em tamanho e tempo de desenvolvimento. Mas, muito provavelmente, esta vantagem por canibalizar irmãos deva ser grandemente mitigada quando há abundância nutricional. Dado que o espaço natural apresenta variação espacial e temporal, a abundância nutricional também é não linear, e isso impactaria na dimensão da vantagem que a realocação de recursos pode proporcionar. Canibalismo é um comportamento muitas vezes situacional (Fox L., 1975), e provavelmente a realocação de recursos propiciada por *S. poulsonii* em *D. melanogaster* também deva ser.

Nossa estimativa de prevalência em Campinas foi bastante baixa, nominalmente zero dentre 251 linhagens analisadas. Somam-se a isso evidências laboratoriais e observações de campo que apontam para a possível tendência de extinção de tal infecção (Ventura *et al.,* 2012; Haselkorn & Jaenike*.,* 2015), bem como a evidência filogenética de transmissões horizontais da infecção no passado (Haselkorn *et al.*, 2009), e temos um panorama que mostra uma teia de complexas e intrincadas interações de parasita, hospedeiro e ambiente. Aqui propomos a perspectiva de que, enquanto realocação de recursos é um mecanismo que potencialmente permita a infecção a perseverar numa população em curto prazo, transmissão horizontal o permitiria a longo prazo, mas mais estudos são necessários para aprofundar tais hipóteses.

6. CONCLUSÃO

Este trabalho aponta, primeiramente, através da baixa estimativa de prevalência de *S. poulsonii* em *D. melanogaster* na cidade de Campinas, que tal infecção potencialmente tenha desaparecido. Essa hipótese se relaciona com a dinâmica complexa de parasita e hospedeiro, e nos leva a pensar em mecanismos que possam ou justificar a ausência atual ou a presença anterior de infecção. Uma das direções, a adotada aqui, é falar sobre a compensação em fitness que a infecção causa ao ter efeito *male-killer*. Esta é uma manifestação do método científico, partindo de observações de campo, cria hipóteses testáveis em laboratório que podem posteriormente ser corroboradas em análises de campo. Além da investigação sobre realocação de recursos via canibalismo, a rota que tomamos, cabe entender que amostras são fotografias, estáticas, da natureza. Para termos melhor compreensão do que de fato ocorre nessa população quanto a essa infecção específica é de fundamental importância que outras coletas sejam realizadas para compreender como a infecção se comporta ao longo do tempo.

Verificamos que canibalismo é amplamente encontrado em *Drosophila,* e que larvas, em diferentes frequências, canibalizam outras larvas e adultos. O canibalismo de ovos foi menos comum e é dependente de tempo e de densidade de canibais, mas também ocorre em *Drosophila*. Mais do que presente, também se observou que canibalismo em *Drosophila* é adaptativo, permitindo que indivíduos chegassem ao estágio adulto exclusivamente via canibalismo competitivo. Tal caráter adaptativo mostrou-se passível de seleção, dado que obtivemos linhagens que canibalizavam mais ou menos através de seleção artificial, mas tal comportamento parece ser desacoplado das vias tradicionais de padrão de alimentação em *Drosophila*. Estudos futuros seriam informativos ao analisar quais regiões genômicas tais linhagens expressam diferencialmente e confirmar a aparente independência do gene *for* e da via metabólica PI3K/Atk. Também seria elucidativo analisar como o tempo afeta a proteção de feromônios em diferentes espécies, dado que ovos podem ser uma importante fonte nutricional nos primeiros estágios larvais.

Demonstramos aqui que a infecção por *S. poulsonii* em *D. melanogaster* acarretou maior tamanho larval, bem como em um menor tempo de desenvolvimento. Estas vantagens em *fitness* ainda precisam ser investigadas quanto a serem resultado

do aumento de canibalismo induzido pela infecção. Sendo este o caso, seriam um mecanismo de manutenção via transmissão vertical em momentos de privação nutricional, muito provavelmente ocorrendo em conjunto com transmissões horizontais, de modo que permanecem duas perguntas correlatas: com que frequência ocorrem transmissões horizontais, e com qual frequência ocorrem cenários onde a escassez alimentar é suficientemente intensa para que seja vantajosa a infecção.

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8. ANEXOS

8.1. DECLARAÇÃO DE BIOÉTICA

UNICAMP

CEUA/Unicamp

Comissão de Ética no Uso de Animais
CEUA/Unicamp

CARTA DE SOLICITAÇÃO – DISPENSA DE ANÁLISE DO PROJETO PELA CEUA/UNICAMP

À CEUA/UNICAMP,

Eu, [Louis Bernard Klaczko], pesquisador responsável pelo projeto de pesquisa, intitulado [Canibalismo e *Spiroplasma* em *Drosophila*], cujo(s) executor(es) são [Louis Bernard Klaczko e Cesar Henrique Mondini], solicito dispensa de análise do referido projeto pela CEUA/UNICAMP.

Justificativa pela qual o projeto foi executado fora da Unicamp e/ou pela qual não há necessidade de análise pela CEUA/UNICAMP (obs.: incluir, caso exista, número e título do protocolo original, aprovado pela CEUA/UNICAMP ou CEUA externa, que geraram os resultados e/ou materiais utilizados): [o material analisado é constituído de moscas do filo Arthropoda e portanto não necessita parecer de ética de acordo com a Lei Arouca Nº 11.794, de 8 de outubro de 2008 Art. 2º, § 2º].
Declaro que não houve/haverá manipulação *in vivo* dentro dos laboratórios credenciados pela CEUA/UNICAMP de animais do subfilo **Vertebrata**.

Louis Bernard Klaczko

Informações importantes:

- Uma cópia do protocolo aprovado/certificado da CEUA externa à Unicamp deverá ser encaminhada juntamente com esta solicitação.
- Este documento também se aplica em caso de doação. Nesse caso, será necessária uma carta de doação do material doado (ex: órgãos, tecidos, ossos, etc) assinada pelo responsável, juntamente com este documento.
- Em casos de materiais (ex: órgãos, tecidos, ossos, etc) comprados em frigoríficos, será necessária a nota fiscal de compra e um atestado sanitário do médico veterinário responsável, juntamente com este documento.

CEUA/UNICAMP
Caixa Postal 6109
13083-970 Campinas, SP – Brasil

Telefone: (19) 3521-6359
E-mail: comisib@unicamp.br
http://www.ib.unicamp.br/ceea/

8.2. DECLARAÇÃO DE DIREITOS AUTORAIS

Declaração

As cópias de artigos de minha autoria ou de minha co-autoria, já publicados ou submetidos para publicação em revistas científicas ou anais de congressos sujeitos a arbitragem, que constam da minha Dissertação/Tese de Mestrado/Doutorado, intitulada **Canibalismo e Spiroplasma em Drosophila**, não infringem os dispositivos da Lei n.° 9.610/98, nem o direito autoral de qualquer editora.

Campinas, 03 de Junho de 2022

Assinatura : _____
Nome do(a) autor(a): **Cesar Henrique Mondini**
RG n.° 46.316.260-2

Assinatura : _____
Nome do(a) orientador(a): **Louis Bernard Klaczko**
RG n.° 55.697.461-X