UNIVERSIDADE ESTADUAL DE CAMPINAS

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**AYANA DE BRITO MARTINS**

# DISTORÇÃO DA PROPORÇÃO SEXUAL INDUZIDA POR *SPIROPLASMA*, UM AGENTE ANDROCIDA, EM *DROSOPHILA*



Dissertação apresentada ao Instituto de Biologia para obtenção do Título de Mestre em Genética e Biologia Molecular, na área de Genética Animal e Evolução

**ORIENTADOR: PROF. DR. LOUIS BERNARD KLACZKO**

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BANCA EXAMINADORA

Prof. Dr. Louis Bernard Klaczko (Orientador)

Assinatura

Profª. Drª. Cláudia Márcia Aparecida Carareto

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“Coro de cor, sombra de som de cor, de mal me quer

De mal me quer, de bem, de bem me diz

De me dizendo assim: serei feliz

Serei feliz de flor, de flor em flor

De samba em samba em som, de vai e vem”

**Tim Maia**

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# Resumo

Elementos citoplasmáticos egoístas (ECEs) são bactérias que apresentam transmissão predominantemente vertical e se mantêm na população hospedeira através do aumento relativo da produção de fêmeas infectadas. Nesses casos a seleção natural favorece mecanismos de manipulação da reprodução do hospedeiro (feminização, partenogênese, mortalidade precoce de machos e incompatibilidade citoplasmática) e, por isso, os ECEs são denominados parasitas reprodutivos. A mortalidade precoce de machos (em inglês *early male-killing*) refere-se à morte da prole masculina devida à presença de um ECE e seus agentes causadores são denominados agentes androcidas. No caso de indutores de feminização, partenogênese e incompatibilidade citoplasmática, o próprio mecanismo de manipulação resulta no aumento relativo na produção de fêmeas infectadas, o que pode explicar a manutenção desses elementos na população hospedeira. Entretanto, para agentes androcidas, o mecanismo de manipulação não resulta automaticamente no aumento da produção de fêmeas infectadas. Uma das hipóteses para explicar a manutenção de agentes androcidas é a realocação de recursos: a morte precoce dos machos libera recursos preferencialmente para as suas irmãs.

Dado que não são conhecidos os mecanismos que explicam a manutenção de agentes androcidas em espécies de *Drosophila*, este mestrado teve como objetivos estimar a prevalência de agentes androcidas em populações de *D. melanogaster* e analisar fatores que podem estar envolvidos com a manutenção desses agentes nessas populações: (i) presença de outros organismos transmitidos verticalmente; (ii) evidências de realocação de recursos em populações naturais e em linhagens no laboratório; e (iii) efeitos em diferentes componentes do valor adaptativo.

A prevalência do fenótipo androcida em populações de *D. melanogaster*, estimada pela contagem de proles, variou entre próxima a 0 e 17,7% e esteve fortemente associada à presença de *Spiroplasma*, detectada por PCR. As razões sexuais das proles de fêmeas infectadas foram heterogêneas, o que sugere variação na expressão do fenótipo androcida. Não foi detectada associação entre o agente androcida *Spiroplasma* e *Wolbachia*, outro ECE que coinfecta populações de *D. melanogaster*.

Foram encontradas evidências consistentes com a hipótese de realocação de recursos em *D. melanogaster*: (i) em experimentos no laboratório, fêmeas infectadas por agente androcida apresentaram menor tempo de desenvolvimento do que fêmeas não infectadas; (ii) e fêmeas do campo infectadas produziram mais filhas em um repique de quatro dias no laboratório. Não houve diferença na produção de filhas entre fêmeas infectadas e não infectadas de uma estirpe padrão do laboratório. É possível que o efeito de *Spiroplasma* em populações naturais esteja associado à composição genética da população hospedeira.

# Abstract

Selfish cytoplasmic elements (SCEs) are maternally inherited bacteria which increase the net production of infected females. Due to a genetic conflict between the SCE and the host genomes, different mechanisms of reproductive manipulation (feminization, parthenogenesis, male-killing and cytoplasmic incompatibility) are favored through natural selection.

For ferminization, parthenogenesis and cytoplasmic incompatibility, the reproductive manipulation by itself results in a greater net production of daughters by the infected females, which may explain the persistence of these elements in the host population. However, this net difference does not hold for male-killer infected and uninfected females. One of the mechanisms that has been proposed to explain the adaptiveness of the male-killing trait is the resource reallocation from dead males to female hosts.

Considering that it is still unclear how male-killers persist in *Drosophila* populations, the present study aimed to assess the male-killer prevalence in *D. melanogaster* populations and to analyze different factors which may explain their persistence in these population: (i) interaction with other vertically transmitted elements; (ii) evidence supporting the resource release hypothesis in natural populations and in laboratory strains; and (iii) direct fitness effects.

The incidence of the male-killing phenotype in *D. melanogaster*, obtained counting the laboratory raised broods of collected females, ranged from close to 0 to 17.7% and was strongly associated with *Spiroplasma* infection, assessed by PCR. The sex ratio of female biased strains had a bimodal distribution which suggests variation in the expression of the male-killing phenotype. No evidence of positive or negative interaction between male-killing *Spiroplasma* and *Wolbachia* (other SCE coinfecting *D. melanogaster*) infections was found.

We found evidence consistent with the resource reallocation hypothesis in *D. melanogaster*: (i) infected females had a shorter generation time in laboratory experiments; (ii) and field females produced more daughters in their first brood in the laboratory. No difference in number of daughter was detected between infected and uninfected females in an experiment using flies from a standard laboratory strain. The effect of male-killing *Spiroplasma* in natural populations may be conditioned to the host's genetic background.

# Introdução

## 1) Elementos citoplasmáticos egoístas

Elementos genéticos egoístas são aqueles que apresentam um padrão de herança diferente da maior parte do genoma de modo a aumentar sua representação na geração seguinte (Werren 1988, Hurst *et al*. 1996, Price e Wedell 2008). Dentre estes há os que apresentam transmissão predominantemente vertical e exclusivamente materna e, por isso, são denominados elementos citoplasmáticos egoístas (ECEs) (Hurst *et al.* 1996).

A seleção natural sobre ECEs favorece mecanismos de manipulação da reprodução do hospedeiro, sendo denominados parasitas reprodutivos (Bandi *et al*. 2001). Esta pressão seletiva advém de um conflito genético entre o genoma dos ECEs, que apresenta herança materna e não mendeliana, e o genoma do hospedeiro, que apresenta herança biparental e mendeliana (Cosmides e Tooby 1981). Estes mecanismos de manipulação reprodutiva incluem: feminização, partenogênese, mortalidade de machos e incompatibilidade citoplasmática (revisão em Bandi *et al*. 2001 e Werren 2008) (Fig. 1).

Incompatibilidade citoplasmática é a diminuição da viabilidade da prole de cruzamentos entre machos infectados e fêmeas não infectadas (revisão em Werren 1997 e em Werren 2008). Esse sistema resulta em uma maior contribuição relativa de fêmeas infectadas para a geração seguinte (revisão em Charlat *et al*. 2003); por isso pode ser usado para promover o espalhamento de genes de interesse em uma população (*e.g*. introdução de transgene que impede a transmissão de um patógeno em uma população de insetos vetores) (revisão em Dobson 2003). Este mecanismo de manipulação é o único que não resulta em desvios na proporção sexual da prole de fêmeas infectadas (revisão em Werren 1997 e em Werren 2008).

A mortalidade de machos (em inglês *male-killing*) refere-se à morte da prole masculina devida à presença de um ECE e seus agentes causadores são denominados agentes androcidas (Montenegro 2001, 2005). Este tipo de manipulação pode ser classificado como precoce, caso ocorra durante a embriogênese, ou tardia, caso ocorra durante ou após o estágio larvar (Hurst *et al*. 1991). Neste trabalho, iremos tratar apenas da mortalidade precoce de machos que é a que resulta em proles com excesso de fêmeas. Além de agentes androcidas, indutores de feminização e de partenogênese atuam como manipuladores da razão sexual (*sex-ratio distorters* em inglês) (revisão em Bandi *et al*. 2001; Hurst e Werren 2001; Charlat *et al*. 2003; Werren 2008).

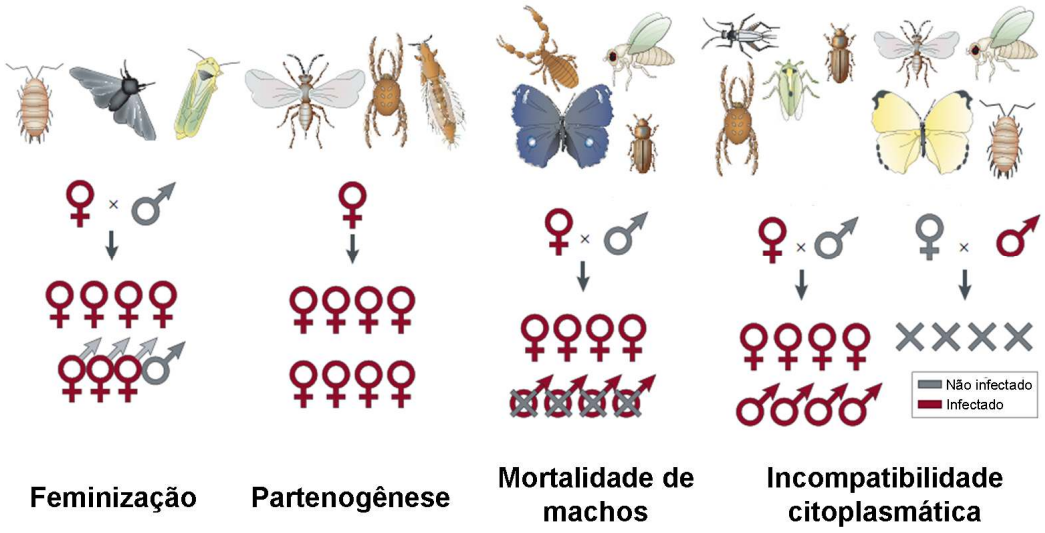


**Figura 1**. A bactéria *Wolbachia* apresenta todas as formas de manipulação reprodutiva descritas para elementos citoplasmáticos egoístas (ECEs). Esta figura ilustra os quatro tipos de manipulação causados por *Wolbachia* e as ordens de artrópodes afetadas. Feminização: Isopoda, Lepidoptera e Hemiptera. Partenogênese: Hymenoptera, Acari e Thysanoptera. Mortalidade de machos: Pseudoscorpiones, Diptera, Lepidoptera e Coleoptera. Incompatibilidade citoplasmática: Orthoptera, Hemiptera, Acari, Coleoptera, Hymenoptera, Diptera, Lepidoptera, Isopoda. Adaptação de Werren 2008.

## 1.1) Agentes androcidas

Agentes androcidas são ECEs que matam a prole masculina de uma fêmea infectada e incluem bactérias de cinco *taxa*: bactérias dos gêneros *Rickettsia* (α-Proteobactéria), *Wolbachia* (α-Proteobactéria) e *Spiroplasma* (Mollicutes), além de *Arsenophonus nasoniae* (γ-Proteobactéria) e bactérias do grupo Flavobactéria. Este tipo de manipulação reprodutiva está presente em diferentes clados de bactérias, por isso acredita-se que tenham ocorrido vários eventos independentes de surgimento de agentes androcidas (revisão em Hurst e Jiggins, 2000).

Diversos modelos têm proposto consequências da presença de agentes androcidas para a dinâmica populacional e evolução de seus hospedeiros (revisão em Hurst e Jiggins 2000). Entretanto, essas consequências geralmente consideram que a prevalência do agente é alta o suficiente para alterar a razão sexual da população (Dyer e Jaenike 2004). Em borboletas das espécies *Acraea encedon* e *Hypolimnas bolina*, a razão sexual de algumas populações é extremamente desviada. A prevalência de *Wolbachia* com fenótipo androcida entre fêmeas destas populações é de 90% e 99% respectivamente (Jiggins *et al*. 2000, Dyson e Hurst 2004). Nestas espécies, algumas das consequências propostas já foram demonstradas.

Populações de *H. bolina* são naturalmente infectadas por *Wolbachia* no Sudeste Asiático. Entretanto, em contraste com outras localidades, fêmeas dessas populações não expressam fenótipo androcida. Hornett e colaboradores (2006) mostraram que a presença de um supressor dominante está associada à não expressão do fenótipo androcida nestas populações. Dados históricos sugerem a presença do efeito androcida em populações do Sudeste Asiático em um passado recente, o que sugere que supressores podem surgir e se espalhar rapidamente na população hospedeira. Além disso, nesta mesma espécie, supressores do fenótipo androcida podem se espalhar rapidamente, promovendo drásticas alterações na razão sexual das populações.

Em alguns casos, a razão sexual da população passou de 99% de fêmeas a 50% de fêmeas em apenas 10 gerações (Charlat *et al*. 2007b).

Agentes androcidas têm transmissão citoplasmática e, por isso, seus genomas podem estar em desequilíbrio de ligação com o genoma mitocondrial de seus hospedeiros. Em *A. encedon*, fêmeas infectadas carregam um haplótipo mitocondrial que sofreu introgressão juntamente com um agente androcida a partir de outra espécie, *A. encedana*. Fêmeas não infectadas de *A. encedon* não tiveram seu genoma mitocondrial alterado pelo espalhamento do agente androcida e apresentam maior diversidade e estruturação geográfica no seu DNA mitocondrial (Jiggins 2003).

Além disso, já foram descritas alterações em comportamentos associados à reprodução: competição entre fêmeas por machos em *Acraea* (Jiggins *et al.* 2000) e aumento da frequência de cópulas em fêmeas e depleção de esperma em machos em *Hypolimnas* (Charlat *et al.* 2007c).

Entretanto, em muitas espécies a prevalência de agentes androcidas é intermediária ou baixa (revisão em Hurst e Jiggins 2000). Nestes casos, o efeito da prevalência da bactéria sobre a razão sexual da população deve ser pequeno. Ainda que a razão sexual difira significativamente de 1:1 em alguns casos (*e.g*. 1,15: 1 em *Adalia bipunctata*; Hurst *et al.* 1993), não se sabe, ao certo, as consequências da presença de agentes androcidas nestes casos (Hurst e Jiggins 2000).

## 2) ECEs em *Drosophila*

Em *Drosophila,* os ECEs encontrados até o momento são bactérias dos gêneros *Wolbachia* e *Spiroplasma* (Mateos *et al*. 2006) que causam mortalidade precoce de machos (agentes androcidas) ou incompatibilidade citoplasmática.

Agentes androcidas já foram descritos em 17 espécies de *Drosophila* pertencentes aos grupos *annulimana*, *cardini*, *guarani*, *quinaria*, *robusta*, *tripunctata* e *virilis* do subgênero *Drosophila* e aos grupos *melanogaster*, *obscura*, *saltans* e *willistoni* do subgênero *Sophophora* (Tabela 1). Além das espécies que apresentam linhagens de *Wolbachia* indutoras de incompatibilidade citoplasmática (Bourtzis *et al*. 1996), algumas apresentam *Wolbachia* ou *Spiroplosma* sem efeito identificado (ver Mateos *et al*. 2006, Haselkorn *et al*. 2009 e Watts *et al*. 2009).

Nosso laboratório foi o primeiro a descrever, a ocorrência natural de agentes androcidas em *D. melanogaster* (Montenegro *et al.* 2000), identificados como bactérias do gênero *Spiroplasma* o que, posteriormente, foi confirmado por um grupo independente (Montenegro *et al.* 2005, Pool *et al.* 2006). Pelo menos duas populações de *D. melanogaster* apresentam *Spiroplasma* (agente androcida) e *Wolbachia* com ocorrência de indivíduos coinfectados (Montenegro *et al*. 2005, Pool *et al*. 2006). Em outra população desta espécie foi descrita a ocorrência de *Spiroplasma* sem efeito identificado, cuja prevalência é de menos de 1% (Watts *et al*. 2009).

**Tabela 1**. Espécies de *Drosophila* nas quais já foram detectados agentes androcidas.

| Espécie | Agente androcida | Referência |
|---|---|---|
| *D. annulimana* | *Wolbachia* | H. Montenegro (comunicação pessoal) |
| *D. aragua* | *Wolbachia* | H. Montenegro (comunicação pessoal) |
| *D. bifasciata* | *Wolbachia* | Ikeda 1970 |
| *D. borealis* | *Wolbachia* | Sheeley e McAllister 2007 |
| *D. equinoxialis* | *Spiroplasma* | Williamson e Poulson 1979 |
| *D. innubila* | *Wolbachia* | Dyer and Jaenike 2004 |
| *D. melanogaster* | *Spiroplasma* | Montenegro *et al.*2005 |
| *D. nebulosa* | *Spiroplasma* | Williamson e Poulson 1979 |
| *D. neocardini* | *Spiroplasma* | Montenegro *et al.* 2006 |
| *D. ornatifrons* | *Spiroplasma* | Montenegro *et al.* 2006 |
| *D. paraguayensis* | *Spiroplasma* | Montenegro *et al.* 2006 |
| *D. paulistorum* | *Spiroplasma* | Williamson e Poulson 1979 |
| *D. prosaltans* | não identificado | Cavalcanti *et al*.1957 |
| *D. robusta* | não é *Spiroplasma* | Poulson 1966 |
| *D. roehrae* | não identificado | A. B. Carvalho (comunicação pessoal) |
| *D. unipunctata* | *Wolbachia* | A. B. Martins (dados não publicados) |
| *D. willistoni* | *Spiroplasma* | Williamson e Poulson 1979 |

## 2.1) *Wolbachia*

A α-proteobactéria *Wolbachia* é a única bactéria a apresentar um espectro tão grande de interações com seus diversos hospedeiros: todas as formas de manipulação reprodutiva descritas para ECEs, além de poder ser mutualista obrigatória. Estima-se que esteja presente em mais 65% das espécies de insetos, além de infectar outros artrópodes e nematódeos (revisão em Werren 2008).

Para as linhagens indutoras de incompatibilidade citoplasmática, o mecanismo de ação parece variar em diferentes hospedeiros (Clark *et al*. 2008). Na vespa *Nasonia vitripennis*, a presença de *Wolbachia* no macho causa assincronia entre o pronúcleo masculino e feminino, resultando na perda dos cromossomos paternos na primeira mitose (Tram *et al*. 2002). Em *Drosophila*, a bactéria pode afetar a expressão de múltiplos genes associados à reprodução e à resposta imune (Xi *et al*. 2008) e sua presença em espermatócitos e/ou espermátides está positivamente correlacionada com a expressão da incompatibilidade citoplasmática (Clark *et al*. 2003). Entretanto, a mesma correlação não foi observada em outros hospedeiros (Clark *et al*. 2008). A bactéria modifica os espermatozóides de machos de *Drosophila* infectados através de um mecanismo desconhecido, resultando em alterações na cinética das primeiras etapas da fertilização e, consequentemente, a primeira clivagem não se completa. Entretanto, quando espermatozóides de machos infectados fertilizam uma fêmea infectada pela mesma linhagem de *Wolbachia*, a sincronia dessas primeiras etapas é restaurada e o embrião se desenvolve normalmente (revisão em McGraw e O'Neill 2004). Neste grupo, o grau de incompatibilidade citoplasmática varia entre espécies (Bourtzis *et al*. 1996).

O sequenciamento completo do genoma da linhagem *w*Mel de *Wolbachia pipientis*, que infecta naturalmente *D. melanogaster*, revelou uma abundância de genes que codificam proteínas

com domínios repetidos de anquirina semelhante a de eucariotos. Este padrão sugere que estes genes devem desempenhar alguma função nos diversos efeitos fenotípicos descritos para os hospedeiros desta bactéria. Comparações com os genomas de α-proteobactérias de vida livre podem ajudar a esclarecer os mecanismos de interação *Wolbachia*-hospedeiro (revisão em McGraw e O'Neill 2004).

O mecanismo de atuação de linhagens androcidas é pouco conhecido, entretanto na borboleta *Hypolimnas bolina* o efeito letal pode ser deslocado para o estágio larval com o uso de antibióticos. Esta alteração temporal sugere que o mecanismo que promove a morte dos machos não atua necessariamente apenas em estágios embrionários quando se dá a determinação do sexo (Charlat *et al*. 2007a).

Há evidências de que os diferentes tipos de manipulação reprodutiva compartilham mecanismos de atuação: uma linhagem de *Wolbachia* que induz incompatibilidade citoplasmática ou feminização em seu hospedeiro natural pode se tornar androcida quando introduzida artificialmente em outra espécie (Fuji *et al*. 2001, Jaenike *et al*. 2007). Além disso, em *Drosophila bifasciata*, os machos que escampam da morte causada por agente androcida apresentam incompatibilidade citoplasmática (Hurst *et al*. 2000).

Em *Drosophila*, *Wolbachia* pode estar presente em diversos tecidos, além dos reprodutivos, onde se dá a transmissão vertical. Tanto a distribuição, quanto o grau de infecção nesses tecidos parece ser particular para cada combinação bactéria-hospedeiro (revisão em McGraw e O'Neill 2004). O efeito desta bactéria sobre o valor adaptativo do hospedeiro pode variar de benéfico a deletério dependendo da combinação entre genoma do hospedeiro, linhagem de *Wolbachia*, localização e densidade de bactérias no hospedeiro e interação com o ambiente (Fry *et al*. 2004, revisão em Brownlie e Johnson 2009). Em *D. melanogaster*, um dos efeitos

benéficos associado a *Wolbachia* é a proteção contra patógenos virais (Hedges *et al*. 2008). Além disso, foi descrita uma transição em 20 anos de parasitismo (efeito negativo sobre a fecundidade) a mutualismo (efeito positivo sobre a fecundidade) em *D. simulans* infectada com *Wolbachia* (Weeks *et al*. 2007).

## *2.2) Spiroplasma*

O gênero *Spiroplasma* é composto por bactérias sem parede celular, helicoidais, que apresentam movimentação ativa e pertencem à classe Mollicutes. A maior parte dos representantes deste gênero é encontrada em associação com insetos. Normalmente, estão presentes no tubo digestório, mas podem também ocorrer na hemolinfa, nas glândulas salivares, nos ovários e na hipoderme (revisão em Regassa e Gasparich 2006).

Alguns spiroplasmas são agentes androcidas, têm transmissão transovariana e causam morte de machos no início do desenvolvimento embrionário antes da gastrulação (Counce e Poulson 1962). Em *Drosophila*, o mecanismo que causa morte dos machos parece estar relacionado à presença de um "*dosage compesation complex*" funcional (Veneti *et al*. 2005). Este complexo ribonucleoproteico promove a hipertranscrição do cromossomo X do macho para equilibrar a expressão de genes deste cromossomo em ambos os sexos (revisão em Gilfillan *et al.*, 2004). A morte dos machos é precedida por apoptose por todo embrião e ocorre logo após a formação deste complexo (Bentley *et al*. 2007).

As filogenias de *Spiroplasma* e espécies hospedeiras de *Drosophila* são incongruentes, sugerindo pelo menos cinco introduções independentes de quatro haplótipos de *Spiroplasma* neste grupo de hospedeiros. Esta incongruência também sugere a ocorrência de transmissão vertical imperfeita e possível transmissão horizontal. Os quatro clados de *Spiroplasma* detectados

em *Drosophila* foram denominados: *tenebrosa*, *ixodetis*, *citri* e *poulsonii*. Esses clados não têm origem monofilética: o clado *poulsonii*, por exemplo, está mais próximo de *S. phoencium*, que ocorre em plantas, do que de outras linhagens presentes em espécies de *Drosophila*. Considerando os dados disponíveis, o fenótipo androcida parece estar restrito ao clado *poulsonii*, compondo um grupo monofilético, mas nem todas as linhagens deste grupo apresentam o fenótipo. Em populações de *D. hydei* foram detectadas duas linhagens não androcidas diferentes pertencentes aos clados *poulsonii* e *citri* (Haselkorn *et al*. 2009).

A presença de *Spiroplasma* já foi descrita em 17 espécies de *Drosophila* (Haselkorn 2010), sendo que em oito casos se tratam de agente androcidas (ver Tabela 1 na página 11). Nos demais casos (*D. aldrichi*, *D. ananassae*, *D. atripex*, *D. hydei*, *D. mojavensis*, *D. neotestacea*, *D. simulans*, *D. tenebrosa* e *D. wheeleri*), as linhagens de *Spiroplasma* não têm efeito conhecido (Haselkorn 2010). Em *D. melanogaster* também foi descrita a ocorrência de *Spiroplasma* não androcida (Watts *et al*. 2009). As prevalências de *Spiroplasma* em *Drosophila* variam de 0,5 a 14% no caso dos agentes androcidas e de 0,4 a 85% no caso dos não androcidas. Prevalências acima de 15% só ocorrem para espécies não androcidas, o que leva ao questionamento do papel dessa manipulação reprodutiva em espécies de *Drosophila* (Haselkorn 2010).

## 3) Dinâmica populacional de agentes androcidas

A maioria das espécies produz machos e fêmeas em números aproximadamente iguais. Em 1930, Fisher apresentou uma explicação evolutiva para este fenômeno: cada indivíduo tem um pai e uma mãe. Se machos e fêmeas não estiverem em mesmo número em uma população, o sucesso reprodutivo *per capita* do sexo mais raro será maior. Sendo assim, genes que promovam um aumento na produção do sexo raro, irão aumentar de frequência até que a razão sexual seja de 1:1. Em 1967, Hamilton estudou a violação dos pressupostos do modelo de Fisher e descreveu situações nas quais a razão sexual de equilíbrio difere de 1:1. Para alguns pressupostos o efeito é mais drástico do que para outros. O modelo de Fisher admite herança biparental e mendeliana e violação deste pressuposto resulta em razões sexuais extremas (Fig. 2).

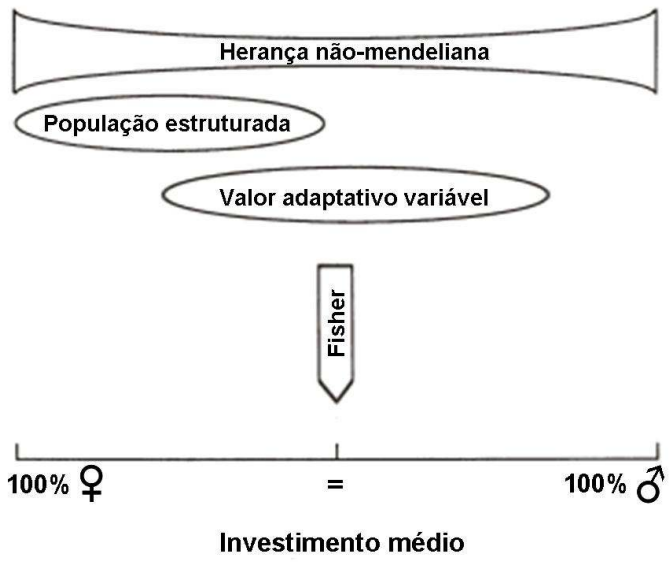


**Figura 2**. O intervalo de razões sexuais plausíveis para cada caso é representado pela proporção do eixo horizontal onde há sobreposição. Se a variação na proporção sexual tiver herança citoplasmática (um caso de herança não-mendeliana), por exemplo, a seleção natural irá favorecer exclusivamente a produção de fêmeas. Adaptação de Bull e Charnov 1988.

Estes desvios extremos previstos na proporção sexual têm como pressuposto a igual contribuição de cada indivíduo para a geração seguinte (Hamilton 1967). Na presença de alguns ECEs ou genes de impulso meiótico, a determinação do sexo está associada a elementos que apresentam herança uniparental e não-mendeliana. No caso de indutores de feminização ou partenogênese, a manipulação reprodutiva em si não interfere na contribuição relativa *per capita* para geração seguinte, pois a produção de machos é redirecionada para a produção de fêmeas. Nestes casos, a seleção natural favorece o aumento da prevalência da infecção e da proporção de fêmeas na população (análogo ao aumento de frequência de gene de impulso meiótico) (Hamilton 1967). Para agentes androcidas, o mecanismo de manipulação não resulta automaticamente no aumento da produção de fêmeas infectadas (Fig. 3), por isso considera-se que a evolução desses agentes deve ser entendida através da seleção de parentesco: para que o agente seja mantido na população é necessário que parentes clonais do elemento suicida localizados nas fêmeas sobreviventes beneficiem-se indiretamente com a morte dos machos (Hurst, 1991)

A morte precoce dos machos pode conferir vantagem para as suas irmãs através da realocação de recursos (Skinner 1985, Hurst *et al.* 1991). Besouros são considerados modelos ideais para o estudo de realocação de recursos, pois as ninfas consomem ovos inviáveis (Hurst *et al.* 1992). Em *Harmonia axyridis* (Coleoptera: Coccinellidae), o consumo de ovos é maior para fêmeas infectadas por agentes androcidas (*Spiroplasma*) do que para fêmeas não infectadas (Nakamura *et al.* 2006). A ecologia da espécie hospedeira é crucial para a hipótese da realocação de recursos: a liberação de recursos deve resultar em aumento no valor adaptativo de irmãs e o recurso liberado deve estar mais disponível para irmãs do que para não-irmãos. Em muitos casos, o papel da realocação de recursos na manutenção de agentes androcidas em populações naturais não é conhecido (revisão em Hurst e Majerus 1993). Este é o caso de espécies em que os ovos

não têm distribuição agregada (*e.g.* algumas espécies de borboletas) ou em que ovos de diferentes fêmeas e espécies ficam agregados (*e.g.* espécies de *Drosophila* que ovipõem em frutos em decomposição) (Hurst e Majerus 1993, McGraw e O'Neill 2007).

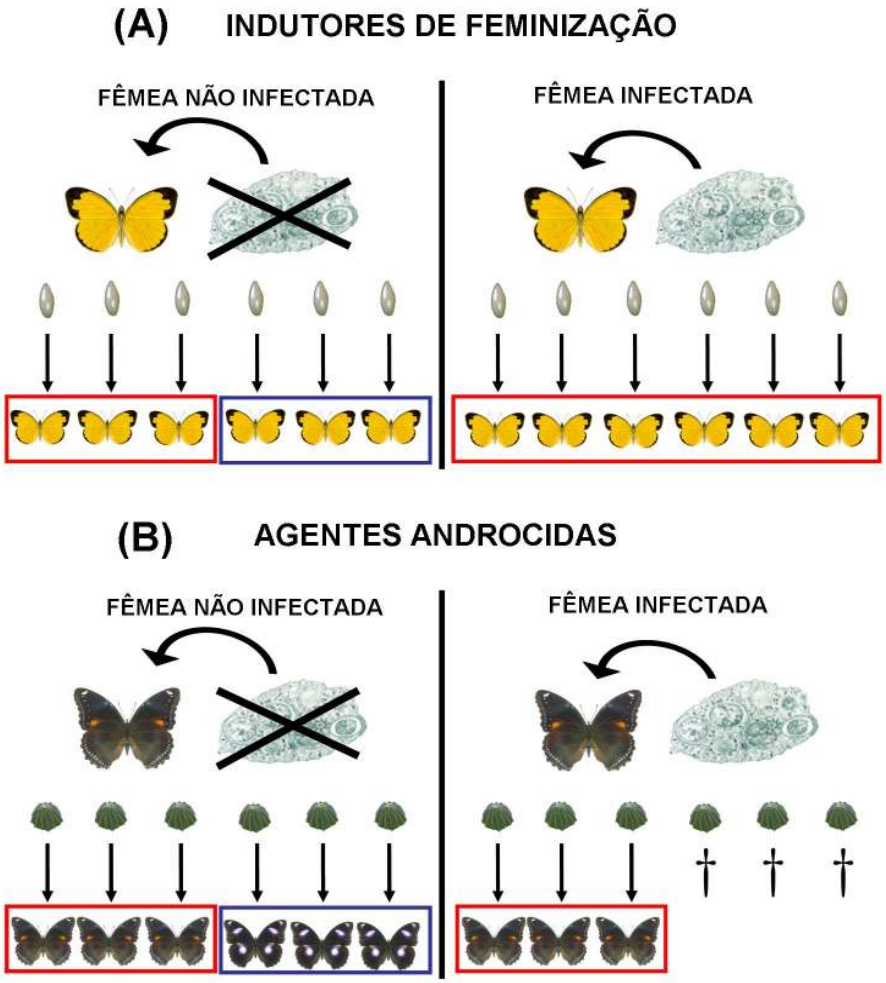


**Figura 3**. **(A)** Esquema ilustrando o efeito de indutores de feminização em *Eurema hecabe*. **(B)** Esquema ilustrando o efeito de agentes androcidas em *Hypolimnas bolina*. Neste caso o mecanismo de manipulação em si não resulta em uma maior produção de fêmeas infectadas. (Diretos sobre as imagens: *Wolbachia* - O'Neill; *Hypolimnas bolina* – Florida Museum of Natural History; ovos de *E. hecabe* e *Hypolimnas* - School of Ecology and Conservation, UAS . As demais imagens são de domínio público).

Diversos fatores operam diminuindo a frequência de agentes androcidas na população hospedeira. Em primeiro lugar, a taxa de transmissão é quase sempre imperfeita (revisão em Hurst e Jiggins 2000 e em Hurst e Majerus 2003). Além disso, temperaturas altas ou baixas e fatores genéticos podem afetar negativamente a taxa de transmissão (Magni 1953, Cavalcanti *et al.* 1957, Malogolowkin e Poulson 1957, Hurst *et al.* 2000, Montenegro e Klaczko 2004, Anbutsu *et al.* 2008, Kageyama *et al.* 2009). Em *D. melanogaster*, a idade da fêmea hospedeira pode alterar a taxa de transmissão e a temporalidade do efeito letal de um agente androcida (Montenegro *et al.* 2000, Kageyama *et al*. 2007). Este fenômeno pode estar associado à densidade da bactéria que apresenta um limiar para a expressão do fenótipo androcida (Hurst *et al.* 2000, Anbutsu e Fukatsu 2003, Dyer *et al.* 2005). Estudos comparativos entre o valor adaptativo de indivíduos infectados e não infectados sugerem a presença de efeitos deletérios em alguns casos (Ikeda 1970, Ebbert 1995). A manutenção desses agentes em populações naturais, apesar da existência de fatores promovendo a sua diminuição, sugere que outros processos devem ser relevantes para a dinâmica desses organismos.

## 4) Objetivos

Dado que não são conhecidos os mecanismos que explicam a manutenção de agentes androcidas em espécies de *Drosophila*, este mestrado teve como objetivos estimar a prevalência de agentes androcidas em populações de *D. melanogaster* (Capítulo 1) e analisar fatores que podem estar envolvidos com a manutenção desses agentes nessas populações: (i) presença de outros organismos transmitidos verticalmente (Capítulo 1), (ii) evidências de realocação de recursos em populações naturais e em linhagens no laboratório (Capítulo 2), e (iii) efeitos em diferentes componentes do valor adaptativo (Capítulo 2).

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# Capítulo 1

## Neutral association between *Wolbachia* and male-killing *Spiroplasma* at the population level

**Ayana de Brito Martins[1], Iuri Mateuzzo Ventura[1], Mariana Lúcio Lyra[2], Carlos Alberto da Conceição Andrade[3], Luciana Ordunha Araripe[4], Klélia Aparecida de Carvalho[1] and Louis Bernard Klaczko[1].**

[1] Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas, São Paulo, Brazil

[2] Laboratório de Genética Animal, Centro de Biologia Molecular e Engenharia Genética, Universidade Estadual de Campinas, São Paulo, Brazil

[3] Departamento de Biologia Marinha, Universidade Federal Fluminense, Niterói, RJ, Brazil

[4] Department of Organismic and Evolutionary Biology, Harvard University, 16 Divinity Avenue, Cambridge, MA 02138, USA

## Abstract

Male killers are maternally transmitted bacteria that kill infected sons to the advantage of infected daughters, resulting in the production of female-biased broods. The prevalence of male killers varies considerably both between and within species and is usually low in *Drosophila*. One of the factors possibly affecting the persistence of these agents is the occurrence of other maternally inherited bacteria in the same population. Mathematical models suggest that the coexistence of multiple infections in host population favors the existence of doubly infected individuals. In *D. melanogaster* male-killing *Spiroplasma* infected females are usually also infected with *Wolbachia*. The interaction of these agents within the host is antagonistic. Here we examined the association between *Wolbachia* and male-killing *Spiroplasma* in a natural population. The incidence of the male-killing phenotype in *D. melanogaster* ranged from close to 0 to 17.7% and was strongly associated with *Spiroplasma* infection. The prevalence of *Wolbachia* ranged from 81.8% to 98.2%, and 100% in a small sample. In our largest sample, the prevalence of *Wolbachia* did not differ between *Spiroplasma* infected and uninfected strains (97.2% and 95.0%, respectively). Therefore, no evidence of positive or negative interaction between *Wolbachia* and male-killing *Spiroplasma* infections was found.

# Introduction

Male killers (**MKs**) are maternally transmitted bacteria that cause death of sons of infected females, resulting in the production of female-biased broods. The death of sons is thought to be advantageous for their female siblings (Hurst and Jiggins 2000, Bandi *et al.* 2001, Werren *et al.* 2008). The prevalence of male-killing bacteria varies considerably both between and within species (reviewed in Hurst and Jiggins 2000), with values ranging from close to 0% in some *Drosophila* species (Williamson and Poulson 1979) to 99% in the butterfly *Hypolimnas bolina* (Dyson and Hurst 2004). Often, the prevalence ranges from 5 to 50% (Bandi *et al.* 2001). In *Drosophila* species the prevalence of male-killing agents is usually low, under 15%, (Malogolowkin 1958, Ikeda 1970, Williamson and Poulson 1979, Montenegro *et al.* 2005) and varies within species among populations separated by few hundred kilometers (Ikeda 1970, Williamson and Poulson 1979). The prevalence of male-killing bacteria can be assessed by two complementary methods: detecting the phenotype by the presence of female-biased broods and detecting the presence of the bacteria by PCR. Both methods are necessary in order to establish the connection between the reproductive manipulation and its causal agent.

Prevalence is primarily determined by three factors: the transmission efficiency of the bacteria from mother to progeny; the effect of infection on female host performance; and the level of advantage to the bacteria conferred by male-killing (Hurst *et al.* 1991, Hurst and Jiggins 2000). In addition, host and/or bacteria genetics and environmental constrains – such as high or low temperatures – may affect **MK** incidence (Magni 1953, Cavalcanti *et al.* 1957, Malogolowkin and Poulson 1957, Hurst *et al.* 2000, Montenegro and Klaczko 2004, Anbutsu *et al.* 2008, Osaka *et al.* 2008, Kageyama *et al.* 2009).

The interaction among multiple maternally inherited selfish genetic elements within the same host is another factor thought to influence their prevalence in host population. Mathematical models suggest interactions between different reproductive manipulators, both between vertically transmitted pathogens (Engelstädter *et al.* 2004a) and between pathogens and meiotic drive chromosomes (Engelstädter *et al.* 2004b). Furthermore, the coexistence of multiple infections in host population favors the existence of doubly infected individuals (Engelstädter *et al.* 2004a, Vautrin *et al.* 2007), which offers possibility for different elements to co-evolve. In the butterfly *Hypolimnas bolina* the variation in **MK** prevalence in natural populations may be explained by interactions with cytoplasmic-incompatibility-inducing *Wolbachia* which can exclude the **MK** from some populations (Charlat *et al.* 2006). Theoretical models, however, suggest that interactions among different reproductive manipulators infecting the same host may range from antagonistic to beneficial (Vautrin *et al.* 2008)

*D. melanogaster* has been one of the most studied organisms since the beginning of the 20$^{th}$ century (Rubin 1988); however the natural occurrence of male-killing agents, identified as bacteria from the genus *Spiroplasma*, has only recently been reported for this species (Montenegro *et al.* 2000, 2005, Pool *et al.* 2006). **MK** infected *D. melanogaster* are usually also infected with *Wolbachia* (Montenegro *et al.* 2005, Pool *et al.* 2006). The only exception so far is the only **MK** infected female sampled in Campinas, Brazil, which tested negative for *Wolbachia* (Montenegro *et al.* 2005). When these agents coinfect *D. melanogaster* their interaction is asymmetrical: *Spiroplasma* negatively affects *Wolbachia*, while the latter does not influence the population of the former (Goto *et al.* 2006). In addition, theoretical models suggest that cytoplasmic-incompatibility-inducing and male-killing strains are mutually antagonistic (Engelstädter *et al.* 2004a). Therefore, a negative association between *Wolbachia* and

*Spiroplasma* infections is expected in natural populations. This association has been difficult to test, because only very few infected flies are normally sampled at a time.

In the present study we assessed the prevalence of male-killing phenotype in *D. melanogaster* in three localities in Brazil counting the laboratory raised broods of collected females, amounting to a total of 326 broods. We also assessed by PCR the prevalence of *Spiroplasma* and *Wolbachia* in *all* female-biased progenies and in control progeny samples, adding up to 191 broods. Due to intensive sampling effort, we were able to test the association between *Wolbachia* and male-killing *Spiroplasma* infections in a natural population.

## Materials and Methods

*D. melanogaster* were collected in January 2008 and January 2009 at Salvador, Bahia State, Brazil; February 2008 at Rio de Janeiro, Rio de Janeiro State, Brazil and March 2009 at Recife, Pernambuco State, Brazil. *D. melanogaster* females were placed individually in small vials containing cornmeal-molasses medium. They were transferred to new vials twice, remaining 4 days in each of the three vials. Then, they were individually stored in alcohol in identified tubes. Only females producing more than 15 descendants or at least 12 daughters were included in the analysis; all animals emerging from each vial were scored. A sample of sons and daughters was separately stored in alcohol. Females which produced female biased offspring sex-ratios (75% of females or more) were considered candidates for **MK** infection (Montenegro *et al.* 2005). Their female descendents were backcrossed to Canton S males for at least two generations in order to confirm the female-biased phenotype. All female-biased strains were tested for *Spiroplasma* and *Wolbachia* infections. All normal strains were also tested, except for the Salvador sample. In this case, 60 randomly chosen strains among the normal and all female-

biased progenies were tested. DNA was collectively extracted from three daughters from each of the tested broods. The infection for *Wolbachia* was assayed using wsp81F and wsp691R primers (Zhou *et al.* 1998); and for *Spiroplasma* SpoulF and SpoulR were used (Montenegro *et al.* 2005). Confidence intervals were calculated using the Adjusted Wald Method (Agresti and Coull 1998).

## Results

The prevalences estimated for all collections are shown in Table 1.

**Table 1**. Prevalence of females producing female-biased strains (over 75% of females) in *D. melanogaster* samples from three Brazilian localities.

| Collection sites | Date | Number of strains (female-biased) | Prevalence (95% CI) |
|---|---|---|---|
| Salvador Bahia State | Jan 2008 | 221 (39) | 17.7% (13.2% – 23.2%) |
| | Jan 2009 | 24 (3) | 12.5% (3.5% – 31.8%) |
| Rio de Janeiro Rio de Janeiro State | Feb 2008 | 59 (1) | 1.7% (<0.01% – 9.9%) |
| Recife Pernambuco State | Mar 2009 | 22 (0) | (0 – 13.0%) |

For the strains collect in January 2008, Salvador, Bahia state, female-biased strains showed a bimodal distribution for sex ratio (proportion of males), while normal strains were statistically homogeneous (Pearson's chi-square, $\chi^2 = 196.26$, d.f. = 181, p = 0.207) (Fig. 1). The sex ratio in the whole sample was 44.3% (7859 females and 6244 males), which is statistically

different from 1:1 ($\chi^2$ = 184.94, d.f. = 1, p < 0.001). Considering only the normal broods (excluding progenies with 75% of females or more), the overall sex ratio was 50.5%, which is not statistically different from 1:1 ($\chi^2$ = 1.33, d.f. = 1, p = 0.249). In January 2009, more flies were collected in the same locality. The proportion of female-biased broods did not differ statistically from the previous year (Pearson's chi-square, $\chi^2$ = 0.44, d.f. = 1, p = 0.525). The sex ratios of normal broods were statistically homogeneous for both Rio de Janeiro (Pearson's chi-square, $\chi^2$ = 68.86, d.f. = 57, p = 0.196) and Recife samples (Pearson's chi-square, $\chi^2$ = 13.02, d.f. = 20, p = 0.877). The sex ratio of the whole sample was of nearly 50.0% at Rio de Janeiro (2117 females and 2119 males) and 47.0% at Recife (539 females and 478 males), which are not statistically different from 1:1 ($\chi^2$ = 0.001, d.f. = 1, p = 0.976; and $\chi^2$ = 3.66, d.f. = 1, p = 0.056, respectively).

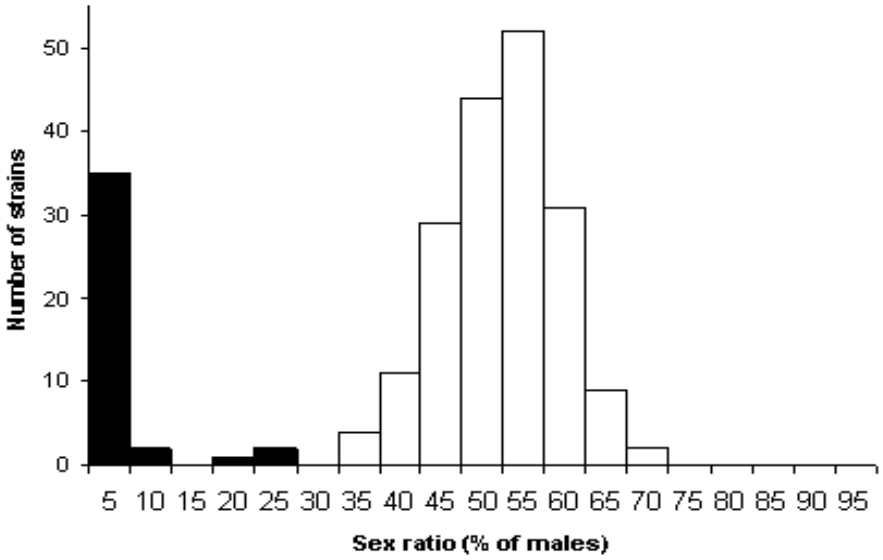


**Figure 1**. Sex ratio (proportion of males) in the $F_1$ of 221 strains from Salvador, Brazil, January 2008. Black: female-biased broods; white: normal broods (≈1:1 sex ratio).

Females from 60 randomly chosen normal broods (≈1:1 sex ratio) from the larger sample from Salvador were tested for *Wolbachia* and *Spiroplasma* infection. Females from 36 of the 39 female biased broods from the same collection were also tested (three progenies were accidentally lost). We found that 33 of the 36 female-biased progenies and three of the 60 normal broods tested positive for *Spiroplasma*, which suggests a tight association between the *Spiroplasma* infection and male-killing phenotype (Fisher's exact test, p = 0.000). The prevalence of *Wolbachia* in *Spiroplasma* infected and uninfected strains is shown in Table 2.

**Table 2**. Prevalence of *Wolbachia* infection in *Spiroplasma* infected and uninfected strains for the sample collect in January 2008, Salvador, Bahia state, Brazil.

| | Positive for *Wolbachia* | Negative for *Wolbachia* | *Wolbachia* Prevalence (95% CI) |
|---|---|---|---|
| *Spiroplasma* uninfected strains | 57 | 3 | 95.0% (85.8% – 98.8%) |
| *Spiroplasma* infected strains | 35 | 1 | 97.2% (84.6% – >99.9%) |

The normal broods which tested positive for *Spiroplasma* (45♀:43♂, 44♀:53♂, 46♀:56♂); as well as the female-biased broods which tested negative for *Spiroplasma* (42♀:9♂, 24♀:0♂, 25♀:8♂), all tested positive for *Wolbachia*. Since three normal broods tested positive for *Spiroplasma* and three female-biased broods tested negative for *Spiroplasma*, the prevalence and confidence interval of *Wolbachia* infection in normal and female-biased strains would be exactly the same as in *Spiroplasma* uninfected and infected strains respectively. There was neither positive nor negative association between *Wolbachia* infection and male-killing

phenotype or *Wolbachia* infection and *Spiroplasma* infection (Fisher's exact test, p = 1.000 for both cases). All female biased broods (n = 4) from other collections tested positive for *Spiroplasma* and all normal broods (n = 105) tested negative. The prevalences of *Wolbachia* estimated for all collections are shown in Table 3.

**Table 3**. Prevalence of *Wolbachia* in *D. melanogaster* samples from three localities.

| Collection sites | Date | Number of strains tested (infected) | *Wolbachia* Prevalence (95% CI) |
|---|---|---|---|
| Salvador Bahia State | Jan 2008 | 96 (92) | 95.8% (89.4% – 98.7%) |
| | Jan 2009 | 16(16) | 100% (82.9% – 100%) |
| Rio de Janeiro Rio de Janeiro State | Feb 2008 | 57(56) | 98.2% (89.8% – >99.9%) |
| Recife Pernambuco State | Mar 2009 | 22(18) | 81.8% (60.9% – 93.3%) |

## Discussion

The incidence of the male-killing phenotype in *D. melanogaster* ranged from close to 0 to 17.7%, which parallels similar estimates for other *Drosophila* species (Malogolowkin 1958, Ikeda 1970, Williamson and Poulson 1979, Montenegro *et al.* 2005). The male killer prevalence in *D. melanogaster* had been previously estimated as 2.3% at Recife, Brazil (Montenegro *et al.* 2005) and 2.6% at Namulonge, Uganda (Pool *et al.* 2006); both estimates are within the range found in this study. The varying prevalence in different collection sites is also in accordance with previous *Drosophila* studies (Ikeda 1970, Williamson and Poulson 1979). While a high prevalence of male-killers can profoundly affect the dynamics of host populations (Hurst and

Jiggins 2000), it is not clear to what extent populations with mid to low prevalence may be affected. Even though, sex ratio distortions are expected to favor the slow increase of sex ratio modifiers favoring the rare sex (Carvalho *et al.* 1998).

The sex ratio of normal strains was homogenous for all collections. Therefore, the distribution of sex ratios may be explained by random fluctuation due to the brood's finite size. In contrast, the sex ratio of female biased strains had a bimodal distribution which is associated with variation in the expression of the male-killing phenotype. This result suggests the presence of expression modifiers in natural populations. Male killer infected *Drosophila* hosts are sensitive to physiological (*e.g*. mother's age), genetic and environmental factors which can affect the expression of male-killing (Magni 1953, Cavalcanti *et al.* 1957, Malogolowkin and Poulson 1957, Hurst *et al.* 2000, Montenegro *et al.* 2000, Montenegro and Klaczko 2004, Anbutsu *et al.* 2008, Kageyama *et al.* 2009).

So far, the natural occurrence of male-killing *Spiroplasma* in *Drosophila* has been reported only in tropical populations of a few species from the subgenus *Sophophora* (Williamson and Poulson 1979, Montenegro *et al*. 2005, Pool *et al*. 2006) and from the *tripunctata* radiation of the subgenus *Drosophila* (Montenegro *et al*. 2006). The sensitivity of male-killing *Spiroplasma* to low temperatures associated with the relative lack of studies including tropical populations may explain why these infections have remained unnoticed for decades.

In natural populations of *D. melanogaster* infection rates of *Wolbachia* have been reported to range from 15% to 85% in Australian (Hoffman *et al.* 1994, Hoffman *et al.* 1998) and Eurasian populations (Illisnky and Zakharov 2006). Montenegro *et al.* (2005) reported an infection rate of *Wolbachia* of 94.4% (34/36) in a sample of *D. melanogaster* from Recife, Brazil.

Our results range from 81.8% to 98.2% of *Wolbachia* infected females. A small sample (n = 16) showed a nominal estimate of 100% but the 95% confidence interval varies from 82.9% to 100%. Actually, a similar case was found by Illisnky and Zakharov (2006) who reported a sample of 17 individuals with 100% *Wolbachia* infection. However, the 95% confidence interval can be estimated as 83.7% to 100%.

Three females which produced female-biased broods tested negative for *Spiroplasma*. Pool *et al*. (2006) reported false negative PCR amplification results while trying to detect *Spiroplasma* using 1 to 2-day-old females of *D. melanogaster*. The same strains tested positive when 7 to 8-week-old females were tested. Male-killing expression (Montenegro *et al.* 2000) and *Spiroplasma* titer (Anbutsu and Fukatsu 2003) increase with female age. Nevertheless, it is advisable to use older females to perform male-killer detection tests. In the present study, 2-week-old females where tested which might still have resulted in few false negatives. In addition, the possibility that other sex-ratio distorters, such as meiotic drive genes (see Jaenike 2001) are responsible for biasing the sex ratio of these broods cannot be ruled out.

On the other hand, three females which produced normal broods tested positive for *Spiroplasma.* Their brood size was relatively large and there are no sign of female excess. Depending on the *Spiroplasma* titer, a strain may express or not the male-killing phenotype (Anbutsu and Fukatsu 2003). It has been proposed that there is a threshold density of male-killer required to the expression of the male-killing trait (Hurst *et al.* 2000, Anbutsu and Fukatsu 2003) and these strains might be bellow this threshold. In addition, non-male-killing *Spiroplasma* has been reported to occur in low frequencies (under 1%) in at least one *D. melanogaster* population in North America (Watts *et al*. 2009).

When two vertically transmitted bacterial genotypes co-exist for several generations, they are expected to evolve towards cooperation due to partner fidelity feedback (Sachs *et al.* 2004). This trend depends on their transmission efficiency (Vautrin *et al.* 2007), if bacteria have a high transmission rate, such as *Wolbachia* (Hoffman *et al.* 1998) and *Spiroplasma* (Hurst and Majerus 1993) infecting *Drosophila*, the benefit of cooperation may not outweigh its costs (Vautrin *et al.* 2007). No evidence of positive or negative interaction between *Wolbachia* and male-killing *Spiroplasma* infections was found in the present study. Goto *et al.* (2006) reported that male-killing *Spiroplasma* negatively affected the *Wolbachia* population within *D. melanogaster* hosts. Apparently, these interactions do not seem to affect the infection status on the population level. However, they used a *D. melanogaster* strain infected with the *Spiroplasma* strain NSRO, originally from *D. nebulosa*, while in the present study naturally infected *D. melanogaster* were used. It is uncertain if the interactions between *Wolbachia* and *Spiroplasma* in the naturally infected hosts are equivalent to the system studied by Goto *et al.* (2006).

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# Capítulo 2

## *Spiroplasma* Infection in *Drosophila melanogaster*: What is the Advantage of Killing Males?

**A. B. Martins[1], I. M. Ventura[1] and L. B. Klaczko[1]**

[1] Departamento de Genética, Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas, São Paulo, Brazil

## Abstract

Male-killing bacteria are maternally inherited agents that cause death of sons of infected females. For this strategy to be adaptive to the male-killer, the bacteria must benefit from the males' death. Their transmission rate is commonly high but imperfect and also sensitive to different environmental factors. Therefore, the proportion of infected females should be reduced in each generation. In order to explain male-killers spread and persistence in host population, a mechanism resulting in the relative increase of infected females must outweigh the losses caused by the imperfect transmission. The resource release hypothesis states that the males' death produces an increase in available resources to sibling females. Infected females are then expected to be larger than uninfected females in natural populations. Alternatively, infected females could have higher viability or shorter development time. Here, we tested the resource release hypothesis by measuring the body size of infected and uninfected wild-caught *Drosophila melanogaster* females and performed other fitness related measures in the laboratory. We have found evidence suggesting resource advantage for infected females resulting in shorter development time or increased female viability. While in our controlled experiment with infected and uninfected flies from a standard laboratory strain no significant difference in the number of daughters was found, wild-caught females produced more daughters in their first days in the laboratory than uninfected females. Fitness effects conditioned to host genetic background are discussed as possible explanation for this difference.

# Introduction

Several maternally inherited parasites manipulate host reproduction increasing the net production of infected females (Bandi *et al.* 2001). Four manipulation mechanisms have been described so far: cytoplasmic incompatibility, feminization of genetic males, induction of parthenogenesis and embryonic male-killing (Bandi *et al.* 2001). For cytoplasmic incompatibility, crosses between uninfected females and infected males are unviable, while all crosses involving infected females are viable. Therefore, infected females gain a relative fitness advantage. The three other cases involve sex-ratio distortion (Werren 2008). If a maternally transmitted parasite evolves the ability to interfere with host's sex determination, female-biased broods will be favoured (Hamilton 1967). For feminization and parthenogenesis the reproductive manipulation by itself results in a greater net production of daughters by the infected females. However, this advantage does not hold for male-killers (Charlat *et al.* 2003). Their transmission rate is commonly high but imperfect, which reduces the proportion of infected females in each generation (reviewed in Hurst and Majerus 1993). Moreover, high and low temperatures may reduce transmission efficiency (Hurst *et al.* 2000, Montenegro and Klaczko 2004, Anbutsu *et al.* 2008, Osaka *et al.* 2008). Therefore, in order to explain male-killers spread and persistence in host population, a mechanism resulting in the relative increase of infected females must outweigh the losses caused by the imperfect transmission.

Among reproductive manipulations, male-killing is particularly widespread, both in relation to host taxa and parasite diversity (Hurst and Jiggins 2000). For this strategy to be adaptive to the male-killing agent, the bacteria present in infected females must benefit from the death of their male siblings **(**Hurst and Majerus 1993). Three main mechanisms have been hypothesized to explain the advantage associated with male-killing: inbreeding avoidance;

horizontal transmission resulting from the male's death; and resource reallocation from dead males to female hosts (reviewed in Hurst and Majerus 1993).

A high degree of heterozygosity and low inbreeding depression has been reported in *D. melanogaster* natural populations (Biémont 1983). The avoidance of inbreeding hypothesis requires that inbreeding is costly and common, so that mechanisms to avoid this cost could evolve. This hypothesis is considered unlikely to explain the persistence of male-killing agents in *Drosophila* species. (reviewed in Hurst and Majerus 1993)

The horizontal transmission hypothesis assumes that the killing of males releases bacteria that are transferred to the uninfected females, e.g. though feeding (Hurst 1991). It is also considered unlikely to explain their persistence in *Drosophila* species, since several attempts to transfer male-killing agents by co-culturing uninfected and infected flies have failed, in spite of a single unconfirmed successful result (Williason and Poulson 1979, Ebbert 1991 and previous attempts reviewed in Carvalho and da Cruz 1962). Phylogenetic evidence suggests the occurrence of horizontal transmission (Sheeley and McAllister 2009, Haselkorn *et al.* 2009). It has been demonstrated that mites can act as vectors for the transmission of male-killing *Spiroplasma* between and within *Drosophila* species (Jaenike *et al.* 2007). However, they transmit bacteria between living flies, which is not dependent of the male-killing. Even though the male-killer persistence in host populations could be explained by horizontal transmission, if mites are acting as vectors, this mechanism would not explain the adaptiveness of male-killing strategy (Hurst and Majerus 1993).

For the resource reallocation hypothesis (Skinner 1985, Hurst 1991) the mechanism involved, in some cases, is very straightforward: female siblings gain inclusive fitness through

the consumption of male dead eggs (Hurst *et al.* 1992). Nakamura *et al.* (2006) demonstrated how this mechanism may occur in *Harmonia axyridis* (Coleoptera: Coccinellidae).

Even for species where sibling cannibalism does not occur, indirect benefits also associated with resource reallocation, such as reduction of competition, could also represent an increase in available resources to sibling females (Hurst and Majerus 1993). It has been demonstrated in laboratory populations that if larval competition is structured, male-killers spread to fixation (Jaenike *et al.* 2003). However, so far, the role of resource allocation as a mechanism maintaining male-killing agents in *Drosophila* natural populations remains unclear. If infected females are gaining inclusive fitness due to their brother's death, they are expected to be larger than uninfected females. Also, resource reallocation could result in a larger number of daughters for infected females in high densities. If there is no competition (or unstructured competition), infected and uninfected females have the same number of daughters. As competition (or the degree of competition structure) increases and viability decreases, the number of surviving individuals becomes limited. Since an uninfected female has both sons and daughters and an infected female has only daughters, the latter is expected to have more daughters in higher densities (Fig. 1). In addition, given similar conditions, the resources released by the males' death could result in reduction of development time of their female siblings.

In order to address these issues, we tested the resource release hypothesis by comparing body size between infected and uninfected wild-caught females. We also compared the number of daughters and development time of infected and uninfected females in two different larval densities in the laboratory. Furthermore, we analyzed number of ovarioles and body size in male-killer infected and uninfected flies in laboratory lines in search of benefits associated with male-killer infection.

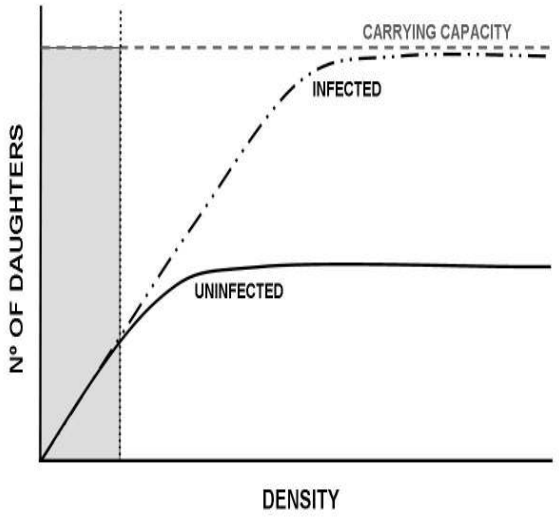



**Figure 1**. Diagrammatic representation of the number of daughters produced by infected and uninfected females as competition (density) increases. Approximately half of the carrying capacity of uninfected lines corresponds to the number of sons.

## Materials and Methods

*Size and number of daughters in wild-caught females*

*D. melanogaster* were collected in January 2008 at Salvador, Bahia State, Brazil. Females were individually placed in vials containing cornmeal-molasses medium for four days at 20ºC. The number of males and females emerging from each vial was scored and a sample of sons and daughters was separately stored in alcohol. Wild-caught females which produced female biased offspring sex-ratios (75% females or more) were considered candidates for male-killer infection (Montenegro *et al.* 2005). DNA was collectively extracted from three daughters from each one of the above mentioned females and they were assayed for *Wolbachia* and *Spiroplasma* infection using SpoulF and SpoulR primers for *Spiroplasma* (Montenegro *et al.* 2005), and wsp81F and wsp691R for *Wolbachia* (Zhou *et al.* 1998). After confirming the male-killer infection, an equal

number of wild-caught females, which produced approximately equal numbers of male and female offspring, were randomly selected to be compared with the male-killer infected ones. They were also assayed for *Wolbachia* and *Spiroplasma* infection following the procedure described above. The thorax length was measured on a lateral view, from the neck to the end of the scutellum from both groups (26 wild-caught females carrying a sex-ratio distorter and 26 randomly chosen wild-caught females which produced ≈1:1 offspring sex-ratio). Also, a daughter from each of these females was randomly chosen and had their thorax length measured.

*Number of daughters and development time in laboratory lines*

The number of daughters produced by infected and uninfected females was assessed at two larval densities. An injected line was obtained through injection of hemolymph from SSB10 adult females into adult Canton-S recipients. The SSB10 strain was collected at Salvador, Brazil in January 2007. Eggs were collected during 12h from mass crosses between 10-day-old infected females from the SSB10 injected line and Canton-S males and from mass crosses between Canton S males and females. The densities used were 16 eggs per vial and 256 eggs per vial (5 replicates each). The eggs were placed in vials containing cornmeal-molasses medium. The number of males and females emerging from each vial was scored every 24 hours until emergence had completely ceased for two days and no pupae or larvae were visible.

*Ovariole number and size in laboratory lines*

Infected first instar larvae were collected from mass crosses between 10-day-old females from the SSB10 line and Canton-S males. The infected strain (SSB10) has been backcrossed to Canton-S males since it has been established from an adult female collected at Salvador, Brazil in

January 2007 (over 14 generations at the time of the experiment). Uninfected first instar larvae were collected from mass crosses between 10-day-old Canton-S females and Canton-S males. The larvae were kept at 22ºC in vials containing cornmeal-molasses medium in groups of 10 with 10 replicates for the infected treatment and 30 replicates for the uninfected treatment. Since mating affects the number of ovarioles (Collinge *et al.* 2006), the size and the number of ovarioles was scored for 30 *virgin* females, aged 10 days, in each treatment. Thorax length, which was used as an estimator for body size, was measured on a lateral view, from the neck to the end of the scutellum. Both ovaries were extracted in distilled water and stained in saturated potassium dichromate for 4 min (Carlson *et al.* 1998, Collinge *et al.* 2006). The dissected ovarioles were teased apart using stainless steel needles and counted. The total number of ovarioles (both ovaries) per female was used to estimate the averages for each treatment (i.e. infected and uninfected). Male-killing bacteria from the genus *Spiroplasma* are transmitted transovarially in *Drosophila* (Counce and Poulson 1962). Therefore, ovariole number is a trait of particular interest in the study of the effects of these agents.

## Results

*Size and number of daughters in wild-caught females*

All female biased broods tested positive for *Spiroplasma* and all normal broods (sex ratio approximately 1:1) tested negative. No significant difference in body size, estimated through thorax length, of infected and uninfected wild-caught flies was found (Table 1). Infected wild-caught females produced more daughters in their first laboratory broods than their uninfected

counterparts. However, uninfected females still had larger broods (males + females). The mean daughter's body size, also estimated through thorax length, was not significantly different either.

**Table 1**. Data (mean ± SE) from wild-caught females. Values for thorax length, number of daughters, number of sons, brood size and daughter's thorax length; and t-tests for the differences between infected and uninfected field flies collected in January 2008 at Salvador, Bahia, Brazil.

| | Infected | Uninfected | |
|---|---|---|---|
| *Thorax length* | 0.91mm ± 0.01 | 0.90mm ± 0.01 | t = 0.70<br>d.f. = 50<br>p = 0.487 |
| *Number of daughters*[*] | 42.54 ± 2.96 | 31.55 ± 0.84 | t = 4.78<br>d.f. = 218<br>p < 0.001 |
| *Number of sons*[*] | 0.64 ± 0.32 | 32.46± 1.02 | t = -29.72<br>d.f. = 208.03<br>p < 0.001 |
| *Brood size*[*] | 43.18± 2.91 | 64.01 ± 1.77 | t = -5.14<br>d.f. = 218<br>p < 0.001 |
| *Daughter's thorax length* | 1,02mm ± 0,01 | 1,03mm ± 0,01 | t = -1.37<br>d.f. = 72<br>p = 0.177 |

[*] in the first 4 days in the laboratory

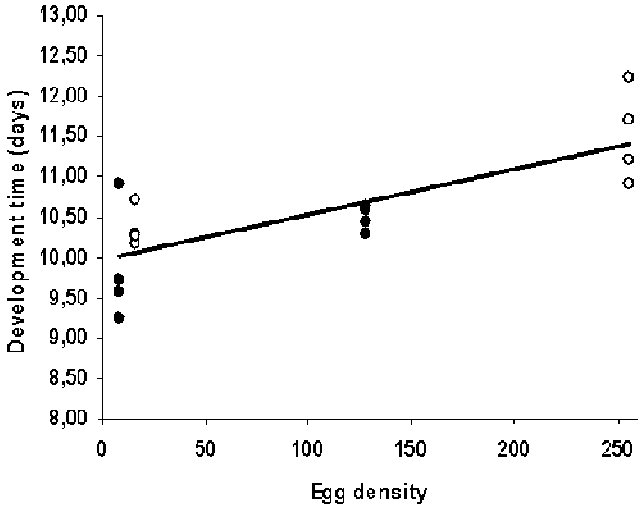
*Number of daughters and development time of infected and uninfected females in laboratory lines*

In contrast with wild flies, the number of daughters did not differ significantly between infected and uninfected females from laboratory lines in both densities assessed. Male-killer infected females produced no sons, leading to a significant difference in brood size (males + females) between infected and uninfected females in both densities (Table 2).

**Table 2**. Viability experiment. Viability values (mean ± SE) are expressed as the number of daughters, number of sons, brood size and development time (days) at two egg densities; and t-tests for the differences between infected and uninfected flies in a Canton-S background.

| | **16 eggs per vial** | | | **256 eggs per vial** | | |
|---|---|---|---|---|---|---|
| | Infected | Uninfected | | Infected | Uninfected | |
| *Number of daughters* | 7.20 ± 1.07 | 8.40 ± 1.44 | t = -0.67 d.f. = 8 p = 0.521 | 55.00 ± 8.84 | 76.40 ± 7.55 | t = -1.84 d.f. = 8 p = 0.103 |
| *Number of sons* | 0 | 9.00 ± 1.10 | | 0 | 70.00 ± 6.37 | |
| *Number of emerging flies* | 7.20 ± 1.07 | 17.40 ± 3.36 | t = -3.94 d.f. = 8 p < 0.01 | 55.00 ± 8.84 | 146.40 ± 13.08 | t = -5.79 d.f. = 8 p < 0.001 |
| *Development time* | 9.83 ± 0.15 | 10.38 ± 0.08 | t = -3.34 d.f. = 76 p < 0.01 | 10.54 ± 0.04 | 11.59 ± 0.05 | t = -15.59 d.f. = 645 p < 0.001 |

Surprisingly, infected females had a shorter development time in both densities than uninfected females (Table 2). In order to assess the relative effect of density and infection status on development time, the density of the infected replicates was considered as half of the number of eggs (since male eggs were unviable due to the male-killing effect), and an ANCOVA was performed with density as a covariate (Figure 2).




| ANCOVA | SS | df | MS | F-ratio | p |
|---|---|---|---|---|---|
| **Infection status** | **4.496** | **1** | **4.496** | **6.421** | **0.011** |
| **Density** | **45.175** | **1** | **45.175** | **64.517** | **0.000** |
| **Infec*Density** | **0.260** | **1** | **0.260** | **0.372** | **0.542** |
| **Error** | **504.846** | **721** | **0.700** | | |

**Figure 2**. Linear regression of development time (days) on egg density (R = 0.769). The density of the infected replicates was considered as half of the number of eggs (since male eggs were unviable due to the male-killing effect). Black dots: infected replicates; white dots: uninfected replicates. ANCOVA showing the effects of infection status and density on development time.

*Size, ovariole number in laboratory lines*

Male-killer infected and uninfected females differed neither in size, estimated through thorax length, nor in ovariole number (Table 4).

**Table 4**. Data from laboratory experiments (mean ± SE). Values obtained for thorax length and ovariole number; and t-tests for the respective differences using infected and uninfected flies in a Canton-S background.

| | Infected | Uninfected | |
|---|---|---|---|
| *Thorax lenght* | 0.95mm ± 0.05 | 0.97mm ± 0.04 | t = -1.46<br>d.f. = 58<br>p = 0.151 |
| *Ovariole number* | 25.23 ± 4.90 | 24.37 ± 3.90 | t = -0.76<br>d.f. = 58<br>p = 0.451 |

# Discussion

Thorax length measures from wild-caught females provided no evidence supporting the resource release hypothesis. The positive correlation between body size and fitness is well known in insects; however competition effects are not always mediated through size (Sørensen and Loeschcke 2001, Baldal *et al.* 2005, Hoffman and Loeschcke 2006).

The significant difference in the number of daughters of infected and uninfected wild-caught females may be a possible evidence of the mechanism by which the male-killing agent is

maintained in *D. melanogaster* host populations. This result could be explained by different phenomena: 1) increased fertility of infected females or differences in the temporal pattern of productivity (since only the first four days after arrival in the laboratory were assessed); 2) differences in daughter viability. The daughters' size did not differ between infected and uninfected females. Any difference in density during larval stage resulting from the sons' death did not affect the daughter's adult size.

No beneficial effect in fertility and/or productivity has been reported for *Drosophila* females naturally infected by male-killing agents (Ikeda 1970, Ebbert 1991, Ebbert 1995, Montenegro *et al.* 2006). Sakaguchi and Poulson (1963) found that Sevelen females of *D. melanogaster* infected with male-killing *Spiroplasma* from *D. willistoni* produced more daughters per day than uninfected females. This result, however, hasn't been confirmed in *D. willistoni* (Ebbert 1991) and in *D. melanogaster* (Montenegro *et al.,* 2006) infected by their naturally occurring male-killing agents. Ebbert (1991) reported that *D. willistoni* infected females produced more offspring in early broods. However, no difference in the temporal pattern of productivity has been reported for *D. melanogaster* (Montenegro *et al.* 2006).

Ovariole number is correlated with the number of eggs produced per day (Boulétreau 1978, Cohet and David 1978). This trait may be considered of particular relevance in the study of male-killing *Spiroplama* because these bacteria are transmitted transovarially (Counce and Poulson 1962). In our study, the ovariole number of infected and uninfected females did not differ, nor did thorax size. Considering the correlation between daily egg production and ovariole number, our results are consistent with previous studies in which no difference in fecundity between infected and uninfected *D. melanogaster* were reported (Montenegro *et al.* 2006).

The increased number of daughters observed could represent an increase in daughters' viability due to resource release from the males' death. We tested this hypothesis by comparing the number of daughters of infected and uninfected *D. melanogaster* females emerging from a given number of eggs (16 or 256 eggs per vial). No significant difference was found. However, in this experiment infected females had a shorter development time, which could result from the reduced competition for infected females, given their male siblings' death. It is widely known that density affects both viability and development time (Moya *et al.* 1986, Zwaan *et al*. 1991). However, its effects on these components are not necessarily linear (Moya *et al.* 1986).

In the experimental conditions used, increased density resulted in increased development time; this effect is clearly present if one compares only the two densities of uninfected eggs (10.38 ± 0.08 and 11.59 ± 0.08). The same result holds true for the infected eggs (9.83± 0.15 and 10.54 ± 0.04). If one assumes that the infected eggs have half the density of the uninfected ones, and all the data are pooled together, then there is a good fit of a linear regression of development time on egg density. This is consistent with the resource reallocation hypothesis explaining the observed difference in development time between infected and uninfected flies as a result of a reduction of egg density.

Nevertheless, the ANCOVA showed that infection by itself still may be at least partially responsible for the differences in development time ($p=0.011$ for infection status). This extra effect was a tenth of the contribution of density to the global variance (mean square = 4.496 and 45.175, respectively). In some cases, vertically transmitted bacteria may have beneficial fitness effects that are independent of the reproductive manipulation (reviewed in Werren 2008 and Brownlie and Johnson 2009). One example is viral protection in *Wolbachia* infected *D. melanogaster* (Hedges *et al.* 2008).

In previous studies, neither *D. bifasciata* infected by male-killing *Wolbachia* nor *D. nebulosa* infected by male-killing *Spiroplasma* presented different development time from their uninfected counterparts (Ikeda 1970, Malogolowkin-Cohen and Rodrigues-Pereira 1975). Fitness effects that could explain a relative increase of infected females in *Drosophila* species in which male-killers naturally occur have been previously reported for two cases. Ebbert (1991) reported that *Spiroplasma* infected females of *D. willistoni* produced more offspring in early broods than uninfected females. Malogolowkin-Cohen and Rodrigues-Pereira (1975) reported that *Spiroplasma* infected females of *D. nebulosa* became receptive to mating earlier than uninfected females. In all cases the fitness effects are not strong. However, the male-killer prevalence for *Drosophila* species are usually low (Malogolowkin 1958, Ikeda 1970, Williamson and Poulson 1979, Montenegro *et al.* 2005) and their transmission rate very high (reviewed in Hurst and Majerus 1993). If one considers that these are equilibrium frequencies, a 1 to 1.2% increase in the fitness of infected females may be enough to explain the male-killer prevalence found in natural populations of *D. melanogaster* (following Werren 1987). Earliness of egg production is a key feature in determining fitness in a continuously breeding population (Lewontin 1974), early emergence as well as early increase in relative fertility and early receptiveness to mating could all be ultimately translated into early egg production. It is worth mentioning that time may be an important factor in the study of the effects male-killing *Spiroplasma* in *Drosophila* hosts.

It has been reported that host genetic background affects the expression of male-killing in *D. prosaltans* (Cavalcanti *et al.* 1957), *D. willistoni* (Ebbert 1991) and *D. melanogaster* which had been infected with the *Spiroplasma* from *D. willistoni* and from *D. nebulosa* (Sakaguchi and Poulson 1963, Kageyama 2009). Furthermore, for *D. melanogaster* infected with *Wolbachia*, the host genetic background can affect the outcome of fitness interactions between bacteria and hosts

(Fry *et al.* 2004). In this study, infected wild-caught females produced more daughters in their first brood in the laboratory than uninfected females. It is still unclear if these differences are due to increased fertility or viability of infected females, or differences in the temporal pattern of productivity, for example. Previously, fitness effects of *Spiroplasma* in *D. melanogaster* had been tested only in Canton S background (Montenegro *et al.* 2006); therefore, the reported results may be possibly associated with different host genetic backgrounds.

The results obtained using both field and laboratory flies are consistent with the hypothesis of resource reallocation either by increasing female viability or by decreasing development time respectively. In addition, the direct effect of male-killing *Spiroplasma* in *Drosophila* development time could not be ruled out. Further experiments assessing fitness effects using different host and bacteria genetic backgrounds will be necessary to better understand the host/male-killer interactions in natural populations.

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# Conclusões

- A prevalência do fenótipo androcida em populações de *D. melanogaster*, estimada pela contagem de proles, variou entre próxima a 0 e 17,7% e esteve fortemente associada à presença de *Spiroplasma*, detectada por PCR. Este intervalo é semelhante ao encontrado em trabalhos anteriores em espécies de *Drosophila*. As razões sexuais das proles de fêmeas infectadas foram heterogêneas, o que sugere variação na expressão do fenótipo androcida.

- Não foi detectada associação positiva ou negativa entre *Wolbachia* e o agente androcida *Spiroplasma* na população estudada. Há evidências na literatura de que *Spiroplasma* interage negativamente com *Wolbachia* nos tecidos de *D. melanogaster*. Caso esteja presente em populações naturais, este efeito parece não interferir na presença dos agentes no nível populacional.

- Foram encontradas evidências consistentes com a hipótese de realocação de recursos em *D. melanogaster*: (i) fêmeas trazidas do campo infectadas produziram mais filhas em um repique de quatro dias no laboratório; (ii) e, em experimentos no laboratório com a estirpe padrão Canton S, fêmeas infectadas por agente androcida apresentaram menor tempo de desenvolvimento do que fêmeas não infectadas, apesar de não haver diferença no número de filhas. Anteriormente, o efeito do agente androcida *Spiroplasma* sobre o valor adaptativo de *D. melanogaster* foi avaliado apenas em linhagens padrão Canton S. É possível que o efeito de *Spiroplasma* em populações naturais esteja associado à composição genética da população hospedeira.