Iuri Matteuzzo Ventura

Prevalência e efeito androcida do endossimbionte *Spiroplasma* em populações de *Drosophila melanogaster*

Orientador: Prof. Dr. Louis Bernard Klaczko

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"PREVALÊNCIA E EFEITO ANDROCIDA DO ENDOSSIMBIONTE *SPIROPLASMA* EM POPULAÇÕES DE *DROSOPHILA MELANOGASTER*"

Tese apresentada ao Instituto de Biologia da UNICAMP 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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À minha avó Paschoa,

pela sabedoria e equilíbrio

Quero dar graças ao Divino
Labirinto dos efeitos e das causas
Pela diversidade das criaturas
Que formam este singular universo,
Pela razão, que não cessará de sonhar
Com um plano do labirinto,
Pelo rosto de Helena e a perseverança de Ulisses,
Pelo amor que nos deixa ver os outros
Tal como os vê a divindade (...)

Jorge Luis Borges, *Outro Poema dos Dons*

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* * *

Índice

Resumo

Elementos citoplasmáticos egoístas são microorganismos de transmissão materna que aumentam sua frequência nas populações através da manipulação da reprodução de seus hospedeiros. Entre as estratégias de manipulação que evoluíram nestes endossimbiontes estão indução de feminização, partenogênese, incompatibilidade citoplasmática e morte precoce dos machos. Como estes microorganismos têm transmissão materna, tais estratégias são selecionadas, pois aumentam a proporção de fêmeas infectadas capazes de transmiti-los. Os fatores mais importantes na determinação da prevalência destes endossimbiontes são a taxa de transmissão, a penetrância do fenótipo induzido e os efeitos benéficos indiretos da infecção. Em espécies de *Drosophila*, os endossimbiontes egoístas descritos até o momento pertencem aos gêneros *Spiroplasma* e *Wolbachia*.

Neste trabalho, estimamos a prevalência das duas bactérias egoístas em populações brasileiras de *D. melanogaster*, determinamos a posição filogenética das linhagens de *Spiroplasma* e verificamos a existência de variabilidade genética para a expressão do efeito androcida entre linhagens de *Spiroplasma* e de *Drosophila* oriundos de diferentes localidades. Desse modo, procuramos estender nossa compreensão sobre os fatores que regulam a dinâmica de *Spiroplasma* nas populações de *D. melanogaster* no Brasil.

A prevalência de *Spiroplasma* androcida variou de 0 a 17,7% (a mais alta estimativa encontrada para uma espécie de *Drosophila*). Também foi observada a ocorrência de Spiroplasma não androcida em uma população, em menor prevalência (3 – 5%), o que pode sugerir uma vantagem de disseminação da estratégia androcida. Todas as linhagens analisadas agrupam-se proximamente no clado de *Spiroplasma poulsonii*. A prevalência de *Wolbachia* foi estimada entre 81 – 100% nas populações amostradas. Não detectamos associação entre as prevalências de *Spiroplasma* e *Wolbachia* no nível populacional.

Em nossos experimentos no laboratório, não foi observada variação no fenótipo androcida induzido por diferentes linhagens de *Spiroplasma*. Por outro lado, detectamos uma variação significativa nas proporções sexuais de diferentes linhagens de *Drosophila* em uma baixa temperatura (18,5ºC). Esta variação provavelmente foi causada por falhas na transmissão vertical do endossimbionte. Não há uma relação clara entre a intensidade do efeito androcida induzido nas linhagens e a prevalência estimada em cada localidade. Porém, uma variação maior foi observada entre linhagens da localidade de Salvador, em comparação com as de Rio de Janeiro. Falhas na transmissão também devem ocorrer em populações naturais, e ajudam a explicar as prevalências baixas e médias observadas para este endossimbionte.

Abstract

Selfish cytoplasmic elements are maternally inherited microorganisms which spread in populations through the reproductive manipulation of their hosts. Several different manipulations of host's reproduction have evolved, including feminization, parthenogenesis, cytoplasmic incompatibility, and male killing. As these microorganisms exhibit maternal transmission, such strategies are selected for, since they increase the proportion of infected females that transmit them. The most important factors for the determination of these endosymbionts prevalence are the transmission rate, the penetrance of the reproductive manipulation and the indirect fitness effects of infection. In *Drosophila* species, the endosymbionts found so far belong to the genera *Spiroplasma* and *Wolbachia.*

In this study, we estimated the prevalence of both selfish bacteria in *D. melanogaster* Brazilian populations, determined the phylogenetic position of *Spiroplasma* strains and verified the presence of genetic variability for the male-killing expression among *Spiroplasma* and *Drosophila* strains collected in different localities. Therefore, we aimed to increase our comprehension of the factors that drive *Spiroplasma* dynamics in *D. melanogaster* Brazilian populations.

The prevalence of male-killing *Spiroplasma* ranged from 0 to 17.7% (the highest estimate found in a *Drosophila* species so far). The presence of non-male-killing *Spiroplasma* was also observed, occurring at lower prevalence (3 – 5%), which may suggest a spreading advantage conferred by the male-killing strategy. All lineages analyzed group closely in the *Spiroplasma poulsonii* clade. *Wolbachia* prevalence varied from 81 – 100% in the populations sampled. We did not detect association between *Spiroplasma* and *Wolbachia* at the population level.

In our laboratory experiments, no variation was observed in the male-killing phenotype induced by different *Spiroplasma* strains. On the other hand, significant variation in the sex ratios was observed among *D. melanogaster* strains at 18.5ºC, probably caused by imperfect transmission of the endosymbiont. There is no clear correlation between male-killing intensity induced in the strains and the prevalence in each population. Nevertheless, greater variation occurred among lineages from Salvador, in comparison with those from Rio de Janeiro. Imperfect transmission or male killing may also occur in the field, and help to explain the low or intermediate prevalences reported for this endosymbiont.

Introdução

Introdução

1. Elementos citoplasmáticos egoístas

Elementos genéticos egoístas são componentes herdáveis que aumentam sua frequência nas populações através da manipulação do padrão de herança mendeliano de seus hospedeiros. Em muitos casos, eles causam a manipulação da reprodução de seus hospedeiros resultando, por exemplo, em desvios nas proporções sexuais (Werren *et al.*, 1988). Nesse contexto, uma categoria especial é constituída pelos elementos citoplasmáticos egoístas (**ECEs**): microorganismos com transmissão materna. A capacidade de induzir diversas estratégias de manipulação reprodutiva evoluiu nestes endossimbiontes, como indução de feminização, partenogênese, incompatibilidade citoplasmática e morte precoce dos machos (*male killing*) (Werren *et al.*, 2008). Os tipos de manipulação reprodutiva que ocorrem em artrópodes estão ilustrados na Figura 1. Como estes microorganismos têm transmissão materna, tais estratégias são positivamente selecionadas, pois aumentam a proporção de fêmeas infectadas capazes de transmiti-los (Hurst & Majerus, 1993). Tais estratégias, portanto, contribuem para a disseminação e persistência dos endossimbiontes nas populações (Unckless & Jaenike, 2012).

Diversos estudos tem demonstrado que bactérias endossimbiontes são muito comuns em populações naturais, especialmente nos artrópodes. Uma estimativa mostrou que mais de 30% das espécies de artrópodes investigadas são infectadas com pelo menos uma das quatro bactérias mais comuns, pertencentes aos gêneros *Arsenophonus, Cardinium*, *Spiroplasma* e *Wolbachia* (Duron *et al.*, 2008). A colonização de novos grupos hospedeiros por estes microorganismos ocorre através de transmissão horizontal (Werren *et al.*, 1995), provavelmente envolvendo ectoparasitas (Jaenike *et al.*, 2007). A ocorrência deste tipo de

transmissão é indicada pela incongruência entre as filogenias dos endossimbiontes e de seus hospedeiros (por exemplo, a infecção de hospedeiros filogeneticamente distantes por bactérias proximamente relacionadas) (Heath *et al*., 1999).

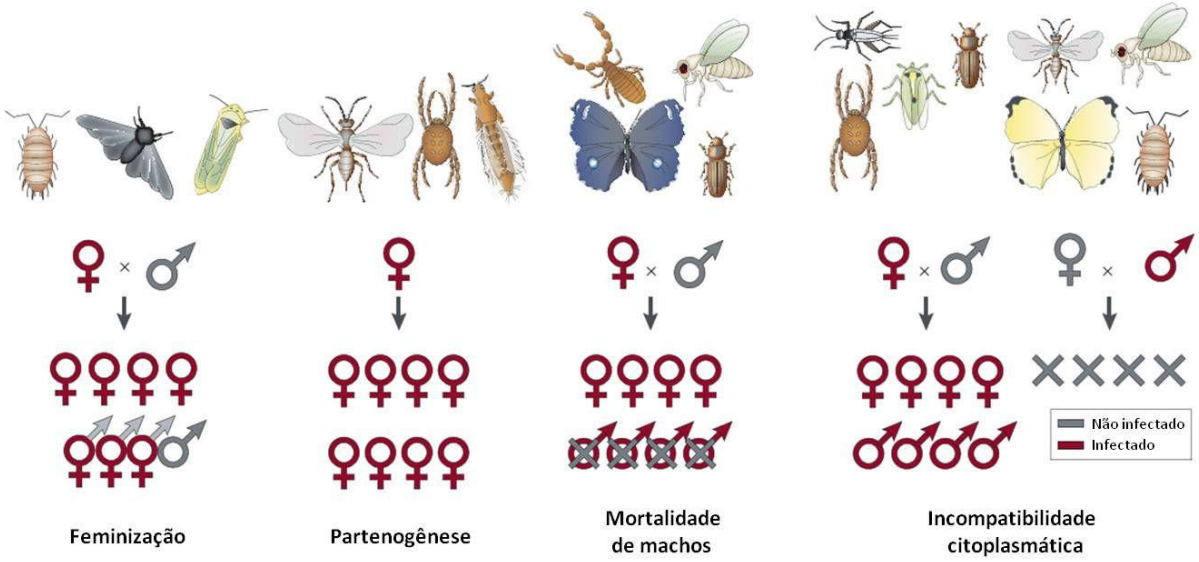

Figura 1: Ilustração das estratégias de manipulação reprodutiva induzidas por ECEs em várias ordens de artrópodes. **Feminização**: Isopoda, Hemiptera e Lepidoptera; **Partenogênese**: Hymenoptera, Acari e Thysanoptera; **Mortalidade de machos**: Pseudoscorpiones, Diptera, Lepidoptera e Coleoptera; **Incompatibilidade citoplasmática**: Orthoptera, Coleoptera, Hymenoptera, Diptera, Acari, Hemiptera, Lepidoptera e Isopoda. Adaptado de Werren *et al*., 2008.

2. Elementos citoplasmáticos egoístas em *Drosophila*

Até o momento, já foi descrita a associação de artrópodes com diversos grupos de microorganismos egoístas, como *Rickettsia*, *Cardinium*, *Arsenophonus*, *Flavobacterium,* Microsporidia (Fungi) e vírus (revisado em Engelstädter & Hurst, 2009). Em espécies de *Drosophila*, os ECEs descritos até o momento restringem-se aos gêneros *Wolbachia* e *Spiroplasma* (Mateos *et al*, 2006).

2.1 *Wolbachia*

A α-proteobactéria *Wolbachia* é encontrada dentro das células nos tecidos reprodutivos dos seus hospedeiros, com os quais pode estabelecer uma ampla variedade de relações, desde parasitismo até mutualismo obrigatório, passando por comensalismo (revisado em Werren *et al.*, 2008). Uma análise mais recente estima que aproximadamente 40% das espécies de artrópodes terrestres são infectadas por *Wolbachia* (Zug & Hammerstein, 2012), o que corresponde a mais de um milhão de espécies.

Em *Drosophila*, *Wolbachia* é capaz de induzir os fenótipos de incompatibilidade citoplasmática e mortalidade de machos. A **incompatibilidade citoplasmática** ocorre quando há diminuição da viabilidade de embriões originados do cruzamento entre fêmeas não infectadas e machos infectados (ilustrada na Figura 1; Rousset *et al.*, 1992). Os mecanismos moleculares envolvidos nesta manipulação ainda são pouco conhecidos, e podem variar de acordo com a espécie hospedeira (Poinsot *et al.*, 2003). Porém, a mortalidade dos embriões deve envolver modificações no esperma dos machos infectados, provocando defeitos nas divisões celulares dos embriões e desenvolvimento assincrônico dos pró-núcleos masculino e feminino. Nos cruzamentos envolvendo machos e fêmeas infectados pela mesma linhagem de *Wolbachia*, a sincronia das divisões é restabelecida e o desenvolvimento embrionário ocorre normalmente (Werren *et al*., 2008). O grau de inviabilidade dos embriões pode variar amplamente em espécies de *Drosophila*, gerando desde proles completamente viáveis até completamente inviáveis (Bourtzis *et al.,* 1996). Com a diminuição da viabilidade da prole de mães não infectadas, o valor adaptativo relativo das fêmeas infectadas aumenta, contribuindo para a disseminação da bactéria nas populações (Engelstädter & Hurst, 2009).

A outra manipulação reprodutiva induzida em *Drosophila* (por *Wolbachia* e *Spiroplasma*) envolve a **mortalidade precoce de machos** (*male killing*). Neste fenótipo, a

prole masculina de uma fêmea infectada é morta em um estágio precoce da embriogênese, o que resulta em proles com excesso de fêmeas (Figura 1; Hurst & Jiggins, 2000). Foi demonstrado que machos de *D. bifasciata* infectados por *Wolbachia* apresentam defeitos na remodelação da cromatina e segregação das cromátides, bem como divisões mitóticas anormais durante o desenvolvimento embrionário (Riparbelli *et al.*, 2012). Acredita-se que a morte seletiva dos machos beneficie as fêmeas infectadas através da redução da competição (realocação de recursos) ou endocruzamento entre elas e seus irmãos (Hurst & Majerus, 1993). Esta estratégia também pode favorecer a dispersão da bactéria androcida nas populações (Unckless & Jaenike, 2012).

2.2 *Spiroplasma*

Os outros endossimbiontes egoístas encontrados em *Drosophila* são bactérias do gênero *Spiroplasma* (classe Mollicutes). Tratam-se de microorganismos helicoidais, com grande motilidade e sem parede celular, presentes dentro e fora das células de seus hospedeiros (Whitcomb, 1981; Gasparich, 2010). A maioria das espécies hospedeiras são insetos, mas aracnídeos e plantas também podem ser infectados (Gasparich *et al.*, 2004). Da mesma forma que *Wolbachia*, estas bactérias podem agir como agentes androcidas, isto é, causar a morte precoce de machos (Williamson & Poulson, 1979). O mecanismo molecular envolvido na indução da morte dos machos de *Drosophila* por *Spiroplasma* foi parcialmente elucidado e difere daquele empregado por *Wolbachia* (Veneti *et al.*, 2005). Ele relaciona-se com o complexo de compensação de dose (*dosage compensation complex*), um complexo ribonucleoprotéico envolvido na hipertranscrição do cromossomo X no macho, necessário para igualar a expressão dos genes deste cromossomo em ambos os sexos (Gelbart & Kuroda, 2009). Esta observação fortalece a hipótese de que diferentes estratégias que causam a morte

seletiva de machos evoluíram independentemente em bactérias androcidas (Riparbelli *et al.*, 2012). Ainda permanecem desconhecidos, porém, os genes envolvidos diretamente na indução da morte dos machos e as diferenças entre linhagens endossimbiontes androcidas e não androcidas (Anbutsu & Fukatsu, 2011).

Até o momento, foram descritas 17 espécies de *Drosophila* infectadas por *Spiroplasma* (sumarizadas na Tabela 1). Em sete delas, o endossimbionte age como agente androcida; em nove ele não tem efeitos sobre a proporção sexual e em apenas uma (*D. melanogaster*), há linhagens androcidas e não-androcidas (Watts et al, 2009; Haselkorn, 2010). Vale ressaltar que, das 17 espécies relatadas como infectadas até o momento, quatro foram encontradas e descritas pela primeira vez por nosso grupo de pesquisa, incluindo o importante modelo *D. melanogaster* (Montenegro *et al.*, 2000; 2005; 2006a).

Tabela 1: Espécies de *Drosophila* infectadas por *Spiroplasma* descritas até o momento. Também estão incluídas a posição filogenética e a prevalência com que o endossimbionte foi encontrado em cada espécie. Adaptado e atualizado de Haselkorn, 2010.

Espécie	Clado de *Spiroplasma* (androcida/não androcida)	Prevalência	Referência
D. melanogaster	*poulsonii* (androcida e não androcida)	0,5 – 3%	Montenegro *et al.*, 2005; Watts *et al.*, 2009
D. simulans	*poulsonii* (não androcida)	0,8%	Watts *et al.*, 2009
D. ananassae	*ixodetis* (não androcida)		Haselkorn *et al.*, 2009
D. atripex	*ixodetis* (não androcida)		Haselkorn *et al.*, 2009
D. willistoni	*poulsonii* (androcida)	< 1%	Williamson & Poulson, 1979
D. paulistorum	? (androcida)	0,4 – 13%	Williamson & Poulson, 1979
D. equinoxialis	? (androcida)	1 – 14%	Williamson & Poulson, 1979
D. nebulosa	*poulsonii* (androcida)	3 – 6%	Williamson & Poulson, 1979
D. hydei	*poulsonii* e *citri* (não androcida)	22 – 66%	Kageyama *et al.*, 2006; Watts *et al.*, 2009
D. aldrichi	*citri* (não androcida)	4%	Watts *et al.*, 2009
D. mojavensis	*citri* (não androcida)	15 – 85%	Watts *et al.*, 2009
D. wheeleri	*citri* (não androcida)	53%	Watts *et al.*, 2009
D. ornatifrons	? (androcida)		Montenegro *et al.*, 2006
D. neocardini	? (androcida)		Montenegro *et al.*, 2006
D. tenebrosa	*tenebrosa* (não androcida)	13%	Watts *et al.*, 2009
D. paraguayensis	? (androcida)	3 – 9%	Montenegro *et al.*, 2006 A.B. Martins (comunicação pessoal)
D. neotestacea	*citri* (não androcida)	10 – 82%	Jaenike *et al.*, 2010

Há grande diversidade genética entre as linhagens de *Spiroplasma* associadas a artrópodes. Entre as espécies de *Drosophila*, há associação com quatro clados diferentes da bactéria (Figura 2). A ausência de correspondência entre a filogenia do endossimbionte e de

espécies de *Drosophila* e outros artrópodes sugere que ocorreram múltiplos eventos de introdução da bactéria nestes gêneros hospedeiros, através de transmissão horizontal (Haselkorn *et al.*, 2009).

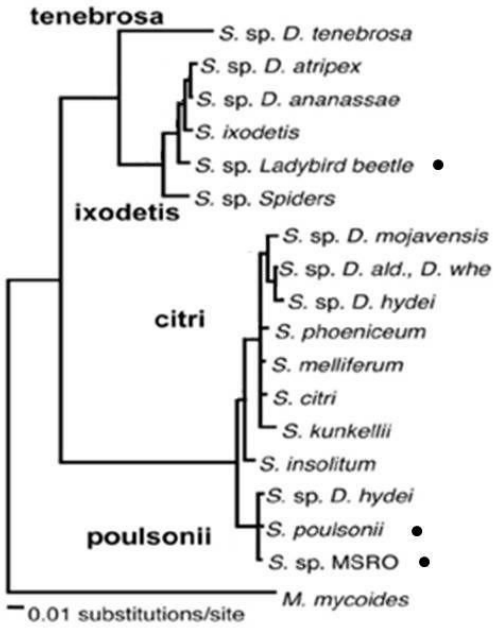

Figura 2: Relações filogenéticas (baseadas no locus ITS) entre linhagens de *Spiroplasma* associadas a diversos hospedeiros. Os quatro clados estão destacados em negrito. Linhagens androcidas estão indicadas por um círculo. Além de linhagens que infectam *Drosophila*, também estão incluídas linhagens encontradas em outros hospedeiros como *S. ixodetis* (que infecta artrópodes), *S. phoeniceum* (plantas), *S. melliferum* (abelhas), *S. citrus* (cítrus), *S. kunkelii* (milho) e *S. insolitum* (plantas e artrópodes). Abreviações: *D. whe.* (*D. wheeleri*), *D. ald.* (*D. aldrichi*), MSRO (*D. melanogaster*). Adaptado de Haselkorn *et al.*, 2009.

3. Dinâmica populacional de elementos citoplasmáticos egoístas

Ainda que o surgimento de novas interações entre ECEs e seus hospedeiros deva envolver mutações em uma linhagem já existente de endossimbionte, gerando novos fenótipos parasitas, a disseminação de uma bactéria egoísta ocorre mais comumente a partir de transferência horizontal para novas espécies hospedeiras (Engelstädter & Hurst, 2009).

Muitos estudos tentaram compreender quais são as condições para que a nova infecção de um endossimbionte egoísta se espalhe, se mantenha, e que prevalência deve ser atingida em uma população (Hoffmann *et al.*, 1990; Hurst, 1991). Análises empíricas e teóricas têm demonstrado que alguns fatores são fundamentais na determinação desta dinâmica: a **taxa de transmissão vertical** dos endossimbiontes (isto é, a proporção dos filhos produzidos por uma fêmea infectada que também carregam a bactéria); a **penetrância do efeito** induzido pelo endossimbionte (por exemplo, a taxa de mortalidade dos machos provocada por um agente androcida); e os possíveis **efeitos benéficos indiretos** causados pela infecção no valor adaptativo (incluindo consequências fenotípicas indiretas nos indivíduos infectados e a vantagem da própria estratégia de manipulação em cada contexto ecológico, isto é, a vantagem da morte dos machos para as fêmeas infectadas da prole, no caso de um agente androcida) (Hurst & Jiggins, 2000; Engelstädter & Hurst, 2009). Abaixo, consideramos cada fator separadamente.

A **taxa de transmissão** de um ECE de uma fêmea infectada para sua prole pode ser imperfeita. As razões das falhas de transmissão são variadas: efeitos ambientais e fatores genéticos do hospedeiro e do endossimbionte podem interromper a transmissão (Hurst *et al.*, 2001; Montenegro & Klaczko, 2004). Além disso, a idade da fêmea infectada também é um fator determinante na taxa de transmissão de agentes androcidas, pois a densidade de bactérias aumenta com o tempo de vida da mosca. Dessa maneira, fêmeas jovens abrigam uma baixa concentração de bactérias, o que faz com que uma proporção de seus ovos não seja infectada e alguns machos consigam sobreviver; porém, com o aumento gradual da concentração de bactérias, um limiar é atingido, a partir do qual todos os ovos são infectados, e a prole passa a ser composta apenas por fêmeas (Anbutsu & Fukatsu, 2003). Nesse contexto, têm-se demonstrado reiteradamente que as condições de temperatura exercem um papel importante na

transmissão de *Spiroplasma*. Em geral, temperaturas acima ou abaixo da ideal diminuem a taxa de transmissão da bactéria (Montenegro & Klaczko, 2004; Anbutsu *et al.*, 2008; Osaka *et al.*, 2008)

A **penetrância do efeito** induzido por um endossimbionte egoísta também pode variar e ter grande influência em sua dinâmica populacional. Alguns estudos já demonstraram que os efeitos induzidos por um ECE dependem da interação entre o background genético do hospedeiro e do endossimbionte. A existência de fatores genéticos que interferem na expressão de parasitas reprodutivos já foi demonstrada tanto entre espécies (Poinsot *et al.*, 1998; Tinsley & Majerus, 2007; Weeks *et al.*, 2007; Hutchence et al, 2012; Veneti *et al.*, 2012) como entre linhagens de uma mesma espécie (Yamada & Watanabe, 1985; Kageyama *et al.*, 2009). Jaenike (2007b) descreve um caso extremo, em que uma linhagem de *Wolbachia* causadora de incompatibilidade citoplasmática em seu hospedeiro natural, *D. recens*, ao ser introduzida experimentalmente em *D. subquinaria*, passou a induzir nela o fenótipo androcida; enquanto outra linhagem da mesma espécie se mostrou resistente à infecção pela bactéria, não exibindo qualquer efeito fenotípico aparente. Este resultado sugere ainda que a indução de morte dos machos e de incompatibilidade citoplasmática devem compartilhar mecanismos moleculares semelhantes. Experimentos deste tipo, com introdução artificial do endossimbionte, demonstram que o tipo e grau da manipulação reprodutiva induzida em um hospedeiro nem sempre podem ser previstos (Engelstädter & Hurst, 2009). Outros estudos já verificaram diferenças mais sutis. Por exemplo, *Wolbachia* pode induzir diferentes níveis de incompatibilidade citoplasmática (Bourtzis *et al.*, 1996) e *Spiroplasma* pode provocar diferentes taxas de mortalidade dos machos (Tinsley & Majerus, 2007), dependendo do hospedeiro que eles infectam. Além disso, a interação entre diferentes endossimbiontes em um

mesmo hospedeiro pode alterar a expressão e a transmissão das bactérias (Charlat *et al.*, 2006; Jaenike *et al.*, 2010).

Por fim, o terceiro fator que influencia a dinâmica de um ECE envolve os **efeitos benéficos indiretos** da infecção para os hospedeiros. Diversos estudos exploraram o impacto da infecção sobre o valor adaptativo dos indivíduos infectados (a maioria utilizando *Wolbachia*). Os resultados são variados: alguns apontaram possíveis efeitos benéficos da interação entre o endossimbionte e o hospedeiro, como proteção contra a infecção por vírus e nematódeos (Hedges *et al.*, 2008; Jaenike *et al.*, 2010b) e diferenças na sobrevivência e fecundidade das fêmeas infectadas (Fry *et al.*, 2004); outros não encontraram nenhum efeito sobre o valor adaptativo dos hospedeiros (Montenegro *et al.*, 2006b); e outros ainda descreveram efeitos negativos da interação, como diminuição do tempo de vida (Min & Benzer, 1997) e da viabilidade de moscas infectadas (Dean *et al.*, 2006). Além deste tipo de consequência sobre o valor adaptativo, também podem existir efeitos advindos da própria vantagem da estratégia de manipulação utilizada em cada contexto ecológico. Por exemplo, a persistência de um agente androcida provavelmente está ligada à realocação de recursos dos machos mortos para suas irmãs infectadas (Koop *et al.*, 2009; Unckless & Jaenike, 2012). Dessa maneira, já foi demonstrado que a estrutura de competição dentro de uma população pode influenciar a vantagem da estratégia androcida empregada por um elemento egoísta (Jaenike *et al.*, 2003).

A prevalência de endossimbiontes egoístas em populações naturais é bastante variável (a Tabela 1 resume valores para *Spiroplasma* em espécies de *Drosophila*), tanto entre espécies quanto entre populações de uma mesma espécie (Watts *et al.*, 2009). Já foram descritos casos em que a prevalência atinge níveis extremos (Dyson & Hurst, 2004), mas, em geral, a frequência de indivíduos infectados é baixa ou intermediária. Na verdade, a dinâmica da

prevalência dos endossimbiontes é governada pela ação simultânea de todos esses fatores, o que pode explicar a ampla variação geográfica e temporal destas bactérias (Jaenike, 2009). É difícil relacionar, porém, a contribuição de cada fator na determinação das prevalências em populações naturais.

4. Elementos citoplasmáticos egoístas e a evolução dos hospedeiros

A assimetria entre a herança de genes nucleares e citoplasmáticos está na base do conflito entre os genomas dos hospedeiros e dos elementos egoístas (Cosmides & Tooby, 1981). Isso ocorre porque, como ECEs têm transmissão exclusivamente materna, os machos representam para eles um "beco sem saída" evolutivo ("*dead end*"). Dessa maneira, como já ressaltado antes, são selecionadas estratégias capazes de aumentar a proporção de fêmeas infectadas em uma população.

Apesar de algumas sugestões, pouco se sabe sobre o impacto que os ECEs podem ter para a evolução e ecologia de seus hospedeiros (Engelstädter & Hurst, 2009). Estudos teóricos e experimentais têm sugerido que as manipulações reprodutivas induzidas por ECEs podem exercer grande influência em vários aspectos da biologia de seus hospedeiros: no tamanho populacional efetivo (Dobson *et al.*, 2002), no sistema reprodutivo (Kuijper & Pen, 2010), na origem de material genético (Hotopp *et al.*, 2007), no fluxo gênico (Telschow *et al.*, 2006), e até nos processos de extinção (Charlat *et al.*, 2003) e surgimento de novas espécies (Jaenike *et al.*, 2006).

Nesse contexto, outra investigação pertinente é a abordagem das interações entre os ECEs e seus hospedeiros como uma corrida armamentista evolutiva, como proposto pela Hipótese da Rainha Vermelha (Van Valen, 1973). Como mencionado anteriormente, os agentes androcidas de transmissão materna são selecionados para aumentar sua transmissão e

expressão, pois assim aumentam a proporção de fêmeas infectadas (Hurst, 1991). Porém, essa manipulação gera o custo da alta mortalidade de machos para a espécie infectada. Assim, aqueles hospedeiros que conseguirem produzir o sexo raro têm seu valor adaptativo aumentado e, portanto, é esperado que genes modificadores e supressores que diminuam a expressão ou transmissão do agente androcida sejam selecionados nos hospedeiros (Jaenike, 2007a). Uma demonstração similar da seleção de supressores contra genes egoístas foi realizada utilizando o sistema cromossômico de impulso sexual (*meiotic drive*) por Carvalho *et al.* (1998), porém, poucos estudos confirmaram as predições no caso de ECEs. Em um caso bem-sucedido, Hornett *et al.* (2006) demonstraram que uma população da borboleta *Hypolimnas bolina* é resistente à ação androcida de *Wolbachia* devido à presença de um único *locus*, que impede a morte dos embriões machos. Trabalhando com a mesma espécie, Charlat *et al.* (2007) relataram que a disseminação de genes supressores provavelmente ocorreu em poucas gerações em uma população natural da Polinésia. Em *D. melanogaster*, foram descritas estirpes de laboratório cujo background genético é capaz de diminuir a expressão do fenótipo androcida induzido por *Spiroplasma* (Kageyama *et al.*, 2009). A localização dos locos supressores, o mecanismo molecular subjacente e a dinâmica em populações naturais, porém, permanecem desconhecidos.

5. Relevância

Estudos envolvendo ECEs têm atraído grande interesse nas últimas décadas (Werren *et al.*, 2008). Em uma revisão recente, Engelstädter & Hurst (2009) destacam que, entre os temas mais promissores a serem investigados nos próximos anos com relação ao assunto, estão: a importância dos benefícios que os endossimbiontes podem oferecer a seus hospedeiros; as relações entre as diferentes estratégias de manipulação reprodutiva; os fatores

que determinam quais espécies e populações são infectadas; e as respostas evolutivas que evoluem nos hospedeiros, como genes supressores. Os dois últimos tópicos estão contemplados neste trabalho. Além disso, a interação entre elementos egoístas e seus hospedeiros é um excelente modelo de estudo das interações entre parasita e hospedeiro, conflito genético e corrida evolutiva.

Destacamos ainda que, além da abordagem dos ECEs como interessante fenômeno biológico, há sugestões da sua utilização no controle biológico de pragas, de maneira análoga à técnica do inseto estéril, na qual a liberação de machos estéreis leva à redução de uma população natural (Zabalou *et al.*, 2004). Estudos recentes envolvendo o vetor da dengue, *Aedes aegypti* (McMeniman *et al.*, 2009; Moreira *et al.*, 2009), bem como o sucesso de algumas iniciativas, corroboram essa visão (Hoffmann *et al.*, 2011). Entretanto, para que essa técnica seja efetivamente empregada, são necessários estudos que melhorem nossa compreensão da dinâmica populacional e dos efeitos evolutivos das interações envolvendo ECEs.

Há alguns anos, nosso grupo tem conduzido pesquisas para a compreensão das interações entre ECEs e populações naturais de *Drosophila* no Brasil. Estas pesquisas iniciaram-se com a primeira descrição da ocorrência de um agente androcida na espécie *D. melanogaster*, um importante modelo animal (Montenegro *et al.*, 2000; 2005). Também foi descrita a ocorrência de *Spiroplasma* androcida em outras três espécies tropicais, *D. ornatifrons*, *D. paraguayensis* e *D. neocardini* (Montenegro *et al.*, 2006).

Além da ocorrência de ECEs entre espécies, várias outras questões pertinentes foram investigadas em populações brasileiras de *Drosophila*, como: efeitos indiretos da infecção sobre o valor adaptativo das moscas (Montenegro *et al.*, 2006); efeitos de condições ambientais sobre a infecção (Montenegro *et al.*, 2004); e a vantagem da estratégia androcida (Martins *et*

al., 2010). Este é um cenário oportuno para verificar a distribuição de ECEs em populações e testar a presença de variabilidade genética entre linhagens de *Spiroplasma* e de *Drosophila*.

6. Objetivos

Como ressaltado anteriormente, não está claro como os diferentes fatores interagem para governar a prevalência de ECEs em populações naturais. Desse modo, este projeto procura estender nossa compreensão sobre os fatores que regulam a dinâmica de *Spiroplasma* nas populações de *D. melanogaster* no Brasil.

Especificamente, pretendemos: 1) estimar a prevalência de ECEs em populações de *D. melanogaster*; 2) verificar a posição filogenética das linhagens de *Spiroplasma*; e 3) verificar a existência de variabilidade genética para a expressão do efeito androcida entre linhagens de *Spiroplasma* e entre linhagens de *Drosophila* oriundas de diferentes localidades, para compreender se fatores genéticos do parasita ou do hospedeiro podem explicar as diferenças observadas nas prevalências das populações brasileiras.

7. Organização da dissertação

Esta dissertação está organizada da seguinte maneira: os dois primeiros objetivos estão reunidos no Artigo 1, que já foi publicado ("*Spiroplasma* in *Drosophila melanogaster* populations: prevalence, male-killing, molecular identification, and no association with *Wolbachia"*). O terceiro objetivo está contemplado no Artigo 2 ("Low temperature reveals genetic variability against male-killing *Spiroplasma* in *Drosophila melanogaster* natural populations"). Após estes capítulos, resumimos nossos principais resultados em uma Conclusão geral.

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Artigo 1

Spiroplasma in *Drosophila melanogaster* populations: prevalence, male-killing, molecular identification, and no association with *Wolbachia*

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Observação: parte dos resultados incluídos neste artigo também se encontra na Dissertação de Mestrado de Ayana de Brito Martins: Martins A.B. (2010). Distorção da proporção sexual induzida por *Spiroplasma*, um agente androcida, em *Drosophila*. Dissertação de Mestrado em Genética e Biologia Molecular – Instituto de Biologia, Universidade Estadual de Campinas.

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INVERTEBRATE MICROBIOLOGY

Spiroplasma in *Drosophila melanogaster* Populations: Prevalence, Male-Killing, Molecular Identification, and No Association with *Wolbachia*

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Abstract *Spiroplasma* endosymbionts are maternally transmitted bacteria that may kill infected sons resulting in the production of female-biased broods. The prevalence of male killers varies considerably both between and within species. Here, we evaluate the spatial and temporal status of male-killing and non-male-killing *Spiroplasma* infection in three Brazilian populations of *Drosophila melanogaster*, nearly a decade after the first occurrence report for this species. The incidence of the male-killing *Spiroplasma* ranged from close to 0 to 17.7 % (so far the highest estimate for a *Drosophila* species) with a suggestion of temporal decline in a population. We also found non-male-killing *Spiroplasma* coexisting in one population at lower prevalence (3–5 %), and we did not detect it in the other two. This may be taken as a suggestion of a spreading advantage conferred by the male-killing strategy. Sequencing two loci, we identified the phylogenetic position of *Spiroplasma* strains from the three localities, showing that all strains group closely in the *poulsonii* clade. Due to intensive sampling effort, we were able to test the association between *Spiroplasma* infections and another widespread endosymbiont, *Wolbachia*, whose prevalence ranged from 81.8 to 100 %. The prevalence of *Wolbachia* did not differ between *Spiroplasma*-infected and uninfected strains in our largest sample nor were the prevalences of the two endosymbionts associated across localities.

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Introduction

Male killers are maternally transmitted bacteria that cause the 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 [2, 20]. The prevalence of male-killing bacteria varies considerably both between and within species [17], with values ranging from close to 0 % in some *Drosophila* species [46] to 99 % in the butterfly *Hypolimnas bolina* [6]. Often, the prevalence ranges from 5 to 50 % [2]. In *Drosophila* species, the prevalence of male-killing agents is usually low, under 15 % [21, 28, 31, 46] (but consistently higher estimates of male-killing *Wolbachia* were found in *Drosophila innubila*, see [5]) and varies among populations separated by few hundred kilometers [21, 46].

The prevalence of male-killing bacteria can be assessed by two complementary methods: detecting the phenotype by the presence of female-biased broods (as well as egg hatch rate) and detecting the presence of the bacteria by PCR.

Both methods are necessary in order to assure the connection between the reproductive manipulation and its causal agent.

Prevalence is primarily determined by two factors: the transmission efficiency of the bacteria from mother to progeny and the net fitness effect of infection on female host performance (including indirect consequences on infected individuals and the advantage conferred by male-killing strategy [17, 19]). In addition, host and/or bacteria genetics and environmental constraints (such as high or low temperatures) can affect those factors and may influence male-killer prevalence [1, 3, 18, 26, 29, 32, 34].

The interaction among multiple maternally inherited selfish genetic elements within the same host is another factor thought to influence their prevalence in a host population [42]. Jaenike et al. [24] report a positive association between *Wolbachia* and *Spiroplasma* in *Drosophila neotestacea*, suggesting a mutualistic interaction. In fact, theoretical models suggest that interactions among different reproductive manipulators infecting the same host may range from antagonistic to beneficial [43]. Likewise, experimental studies have found variable interactions in the field [41, 45].

D. melanogaster has been one of the most studied organisms since the beginning of the twentieth century [38]. However, only recently the natural occurrence of male-killing agents was reported by our group in a Brazilian population of this species [30]. Later on, the male-killing agent was identified as *Spiroplasma poulsonii* and independently confirmed in an African population [31, 35]. There is a large genetic diversity among the *Spiroplasma* found in *Drosophila*, including strains that are not male killers, whose prevalences are also variable and may reach high levels [25, 44]. It was demonstrated that the introduction of the endosymbiont into *Drosophila* species occurred several times [13], probably carried by mites [23]. *Spiroplasma*-infected *D. melanogaster* are usually also infected with *Wolbachia* [31, 35]. So far, the only exception is a single male-killer-infected female sampled in Campinas, Brazil, which when tested was negative for *Wolbachia* [31]. When these agents coinfect the same *D. melanogaster* individual, their interaction may be asymmetrical: *Spiroplasma* can negatively affect *Wolbachia* density within the host, while the latter does not influence the population of the former [11]. In addition, theoretical models suggest that cytoplasmic incompatibility-inducing and male-killing strains are mutually antagonistic [8]. Therefore, a negative association between *Wolbachia* and *Spiroplasma* infections is expected in natural populations. This association has been difficult to test because only 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, as well as *Spiroplasma* and *Wolbachia* prevalences. Due to intensive sampling effort, we were able to test the association between *Wolbachia* and *Spiroplasma* infections. Furthermore, we identify the phylogenetic position of male-killing and non-male-killing *Spiroplasma* strains from the three localities through sequencing of two loci. Therefore, we evaluate the spatial and temporal status of *Spiroplasma* infection in natural populations of *D. melanogaster*, nearly a decade after its first finding in this species, in Brazil [30].

Methods

D. melanogaster were collected in January 2008, 2009, and 2010 at Salvador (12°58′ S 38°30′ W), Bahia State; in February 2008 at Rio de Janeiro (22°54′ S 43°12′ W), Rio de Janeiro State; and in March 2009 at Recife (08°03′ S 34° 52′ W), Pernambuco State, all in Brazil. 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 at −19 °C in labeled tubes. Only females producing more than 15 offsprings or at least 12 daughters were included in the analysis, and all animals emerging from each vial were scored. A sample of sons and daughters was separately stored in alcohol. Females that produced female-biased offspring sex ratios (75 % of females or more) were considered candidates for male-killer infection [31]. In order to confirm the non-male-killing condition of *Spiroplasma* infecting two sampled fly strains, the hemolymph of infected females was injected into virgin females, whose offspring sexual proportion was then analyzed (adapted from [30]). All female-biased strains were tested for *Spiroplasma* and *Wolbachia* infections. All normal strains were also tested, except for the Salvador sample of 2008 (due to the large size of the 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 [48]; for *Spiroplasma*, SpoulF and SpoulR were used [31]. Negative and positive controls (Canton-S and RED42 strains, respectively [31]) were used on DNA extractions and PCR.

In order to infer the phylogenetic position of the *Spiroplasma* collected in Brazil, a sample of 11 strains had the PCR products sequenced for two loci: a highly conserved and a rapidly evolving one (16S ribosomal RNA and *fruR*, partial fructose operon, respectively). From Rio de Janeiro, strains NIA5, RCH2, and RLB27 were analyzed; from Recife, strains RED42, RELH57, and REN27; and from Salvador, strains SSB10, SSG190, SSJ38, SSJ6, and SSJ89 (the last two are non-male-killing *Spiroplasma*). For the 16S rRNA, the amplification was carried out using primers Spiro16SF (GAC GGT CTT CGG ATT GTA AAG GTC TG) and Spiro16SR (GTA CCG GCC ATT

GTA GCA CGT GTG) with an initial denaturation temperature of 94 °C for 5 min, followed by 35 cycles of 1 min at 94 °C, 1 min at 56 °C, and 1 min at 72 °C; for the *fruR* locus, primers fru-f and fru-r were used following that of Montenegro et al. [31]. The amplified products were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) and sequenced bidirectionally using the amplification primers in an ABI Prism 3700 DNA sequencer at Centro de Biologia Molecular e Engenharia Genética, Unicamp. The sequences were edited using Chromas 2.33 software and aligned using ClustalW [40]. The most closely related *Spiroplasma* sequences were downloaded from the National Center for Biotechnology Information. Models of molecular evolution were chosen using Akaike information criteria in Modeltest 3.7 [36]. For the 16S rRNA locus, the model selected was F81 and for the *fruR*, model K81uf. The phylogenetic analyses were performed under Bayesian inference, using Mr. Bayes 3.1.2 [37]. Two independent Markov chain Monte Carlo runs were performed, with 1 million generations each. Trees were sampled every 100 generations, and the first 2,500 trees were discarded as burn-in.

After making sure that the trees produced by the two genes were congruent (Icong index>1.99; $p<0.01$ [4]), a concatenated tree was constructed based on the combination of both loci, using the topology with the highest posterior probability.

Results

Prevalence of Female-Biased Strains, *Spiroplasma* and *Wolbachia*

The prevalences of female-biased strains estimated for all collections are shown in Table 1. For the collection in January 2008, Salvador, the proportions of males in the progenies of collected females and the *Spiroplasma* screening results are shown in Fig. 1. The four black columns on the left represent 39 strains that produced distorted sex ratios; among them, 34 strains (the first column) did not have a single male in their progenies, showing a strong male-killing phenotype. The two black columns at 45 and 50 % males represent the normal broods carrying non-male-killing *Spiroplasma* found in this sample. Normal strains were statistically homogeneous (Pearson's chi-square, $\chi^2=196.26$, d.f. = 181, $p=0.207$) and had sex ratio of 50.5 %, which is not statistically different from 1:1 ($\chi^2=1.33$, d.f. = 1, $p=0.249$). The sex ratio in the whole sample was 44.3 % (7,859 females and 6,244 males), which is statistically different from 1:1 ($\chi^2=184.94$, d.f. = 1, $p<0.001$). Each individual female-biased brood was also tested independently and deviated significantly ($p<0.05$) from 1:1 (data not shown). In January 2009 and 2010, more flies were collected in the same locality.

The proportion of female-biased broods did not differ statistically among the three years ($\chi^2=2.90$, d.f. = 2, $p=0.23$), and the sex ratio of the whole sample was again different from 1:1 (44 % for 2009, $\chi^2=11.21$, d.f. = 1; and 46 % for 2010, $\chi^2=54.75$, d.f. = 1, $p<0.001$ for both cases). A logistic regression, although not significant, suggests a decrease of the prevalences across the 3 years ($R^2=0.99$, $p=0.08$, d.f. = 1). The sex ratio of the whole sample was nearly 50.0 % at Rio de Janeiro (2,117 females and 2,119 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). The sex ratios of normal broods were also statistically homogeneous for both Rio de Janeiro ($\chi^2=65.86$, d.f. = 57, $p=0.196$) and Recife samples ($\chi^2=29.62$, d.f. = 21, $p=0.10$). The proportion of female-biased strains differed significantly between the largest sample from Salvador and Rio de Janeiro (Fisher's exact test, $p<0.001$) and between the largest sample from Salvador and Recife (Fisher's exact test, $p<0.05$), whereas the proportion of distorted strains did not differ between Rio de Janeiro and Recife (Fisher's exact test, $p=1$). No significant correlation was found between *Spiroplasma* prevalence and minimum, average, and

Table 1 Prevalence of females producing female-biased strains (broods with more than 75 % of females) in *D. melanogaster* samples from three Brazilian localities, with the respective average temperatures in the month of collection

Collection sites	Date	Number of strains (female-biased)	Prevalence (95 % CI)	Average temperature (min; avg; max °C)
Salvador, Bahia State	Jan 2008	221 (39)	17.7 % (13.2–23.2 %)	23; 26; 29
	Jan 2009	24 (3)	12.5 % (3.5–31.8 %)	25; 27; 30
	Feb 2010	73 (7)	9.6 % (4.5–18.8 %)	25; 28; 31
Rio de Janeiro, Rio de Janeiro State	Feb 2008	59 (1)	1.7 % (<0.01–9.9 %)	22; 26; 31
Recife, Pernambuco State	Jan 2003[a]	173 (4)	2.3 % (0.6–5.8 %)	26; 28; 31
	Mar 2009	22 (0)	0 % (0–13.0 %)	25; 28; 31

All biased strains were infected with *Spiroplasma*

[a] Data from [31]

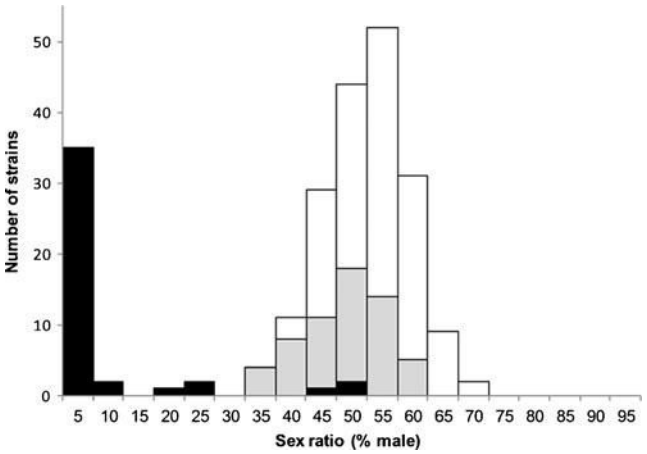

Figure 1 Sex ratio (proportion of males) in the F_1 of 221 strains from Salvador, Brazil, in January 2008. Colors indicate the result of PCR screening for *Spiroplasma*: *black* positive, *gray* negative, and *white* untested broods

maximum temperatures of the month of collection (r=−0.15, −0.41, and −0.78; p=0.78, 0.42, and 0.07, respectively). These tests are not powerful since there is little variation in temperature among localities. Nevertheless, a large variation of prevalences occurs among localities with small variation of temperatures, suggesting that it is very unlikely that temperature variation causes the variation among sites.

For all collections, the proportion of females in the biased progenies was very high. In each collection from Salvador, the proportion of females was 98.0, 100, and 98.5 %, respectively; from Recife, it was 98.6 %; and from Rio de Janeiro, it was 100 %. These high proportions indicate high penetrance of the male-killing effect and high transmission rate of the endosymbiont (although it was previously demonstrated that cultivated *Spiroplasma* may induce male death without vertical transmission [47], we confirmed its presence by PCR in F1 females of all biased strains).

Females from 60 randomly chosen normal broods (≈1:1 sex ratio) from the sample from Salvador in 2008 were tested for *Wolbachia* and *Spiroplasma* infection. Thirty-six females of the 39 female-biased broods from the same collection were also tested (three progenies were accidentally lost). We found that all 36 female-biased progenies tested were positive for *Spiroplasma*, which suggests a tight association between the male-killing phenotype and *Spiroplasma* infection (Fisher's exact test, p<0.001). Actually, in a first trial, three strains failed to give a positive PCR result; but upon retesting, all confirmed the *Spiroplasma* infection. Two of these three strains had an incomplete male-killing phenotype, although clearly sex ratio-biased (75.8 and 82.4 % of females in the progenies). Thus, the initial failure of amplification may have been due to low bacterial density in the first females tested or simply human error.

In the sample from Salvador in 2008, the prevalence of *Wolbachia* did not differ in *Spiroplasma*-infected and uninfected strains (Table 2). Therefore, there was neither positive nor negative association between *Wolbachia* and *Spiroplasma* infections (Fisher's exact test, p=0.65). This test is not very powerful since *Wolbachia* prevalence is very high. A different approach is to test whether there is a correlation between the *Spiroplasma* and *Wolbachia* prevalences among the sampled localities. The prevalences of *Wolbachia* estimated for all collections are shown in Table 3. As the previous test suggested, there is no evidence of correlation between the endosymbionts prevalences (Pearson's correlation, r=0.59, p=0.21, d.f. = 4).

Prevalence of Non-Male-Killing *Spiroplasma*

Three of the 60 normal broods (5.0 %; 95 % CI: 1.2–14.3 %) in the sample from Salvador in 2008 (see Fig. 1) and two of the 66 normal broods (3.0 %; 95 % CI: 0.2–11.0 %) from Salvador in 2010 were positive for *Spiroplasma*. In the last case, the two strains have been maintained in the laboratory, and both male and female from the progeny are infected. The hemolymph of two infected females was successfully injected into females from a standard Canton-S strain (which allows strong male-killing expression [26]). The infection had no effect on the infected offspring (PCR-confirmed) sex ratio (female proportion=53 and 49 %; n=766 and n=1,317, respectively). This demonstrates that the *Spiroplasma* carried is from a non-male-killing strain, discarding the fly's genetic background as the cause of the absence of sexual distortion in the sampled flies. Conservatively assuming the absence of coinfection, the prevalence of the non-male-killing bacteria is significantly lower than that of the male-killing for the Salvador sample in 2008 (Fisher's exact test, p<0.05) and not significantly different in 2010 (Fisher's exact test, p=0.17). All female-biased broods (n=54) tested were positive for

Table 2 Prevalence of *Wolbachia* infection in *Spiroplasma*-infected and uninfected strains for the sample collected in January 2008 in Salvador

	Positive for *Wolbachia*	Negative for *Wolbachia*	*Wolbachia* prevalence (95 % CI)
Spiroplasma uninfected strains	57	3	95.0 % (85.8–98.8 %)
Spiroplasma-infected strains	38	1	97.4 % (85.6 – >99.9 %)

Table 3 Prevalence of *Wolbachia* in *D. melanogaster* samples from three Brazilian 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 %)
	Feb 2010	73 (68)	93.1 % (84.6–97.4 %)
Rio de Janeiro, Rio de Janeiro State	Feb 2008	57 (56)	98.2 % (89.8–100 %)
Recife, Pernambuco State	Jan 2003[a]	36 (34)	94.4 % (80.9–99.4 %)
	Mar 2009	22 (18)	81.8 % (60.9–93.3 %)

[a]Data from [31]

Spiroplasma, and all normal broods from other collections tested were negative (n=270).

Phylogenetic Position of *Spiroplasma*

The concatenated phylogenetic tree based on 365 bp of 16S rRNA and 329 bp of the *fruR* is shown in Fig. 2. The phylogenies for each gene separately are in the supplementary material (Figs. S1 and S2). All the sequenced *Spiroplasma* infecting *D. melanogaster* fall into the *poulsonii* clade, including the non-male-killing strains. All strains are identical to the previous reported *Spiroplasma poulsonii* infecting *D. melanogaster* in Brazil [31] and Uganda [35], as well as that infecting *D. nebulosa*. Within the *poulsonii* clade, *Spiroplasma* infecting *Drosophila hydei* groups separately. As outgroups, *Spiroplasma* sequences in the *citri* clade are shown.

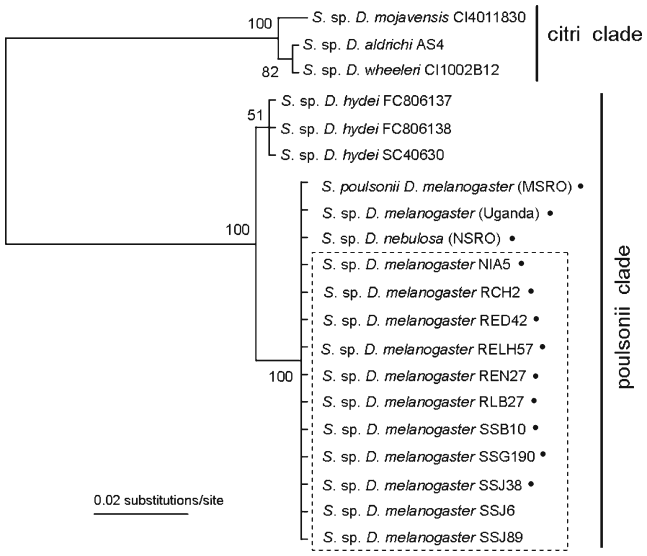

Figure 2 Bayesian phylogeny based on concatenated sequences of 16S rRNA and *fruR* loci of *Spiroplasma* infecting *Drosophila* species. Support for clades given as Bayesian posterior probabilities. Strains sequenced in this study are enclosed by a *dashed rectangle*. Male-killing strains are highlighted by a *small circle*

Discussion

Spiroplasma and *Wolbachia* Prevalences

The prevalence of the male-killing *Spiroplasma* in *D. melanogaster* ranged close from 0 to 17.7 %, which parallels similar estimates for other *Drosophila* species [28, 31, 46]. It is worth noting that a prevalence of 17.7 % of infected females in the largest sample from Salvador is the highest estimate found in a *Drosophila* species (*Spiroplasma* prevalences were recently summarized by Haselkorn [12]). The male-killing *Spiroplasma* in *D. melanogaster* had been previously estimated as 2.6 % at Namulonge, Uganda [35], which is within the range found in this study. *Wolbachia* reached higher and more stable prevalences across localities (from 81.8 to 100 % of infected females), possibly due to one or a combination of the following factors: high transmission fidelity; induction of cytoplasmic incompatibility, even if weak [9]; and beneficial effects on infected female fitness (such as increased fecundity and virus protection [10, 14]), although no effects were found in a study using flies and endosymbionts from the same geographical area [33]. In natural populations of *D. melanogaster* infection rates of *Wolbachia* have been reported to range from 15 % to 85 % in populations from Australia [15, 16] and Eurasia [22]. The estimates of *Wolbachia* prevalence as 92.7 % in 2003 and 81.8 % in 2009 in the Recife population indicate that the bacteria have been maintained in high prevalence for at least 6 years ([31] and this work).

The sex ratios estimated for Salvador in 3 years indicate that the male-killing *Spiroplasma* prevalence in this locality is sufficient to cause a significant distortion from the 1:1 proportion in the progenies born in the laboratory (44 % male in the most distorted sample against 50 % in the 2008 sample of Rio de Janeiro). While a strong sexual distortion can profoundly affect the dynamics of host populations [17], it is not clear to what extent populations with subtle sex ratio distortion may be affected.

The sex ratio of normal strains was homogenous for all collections. Therefore, the distribution of their sex ratios may be explained by random fluctuation due to the brood's

finite size. For the female-biased strains, the great majority (34 out of 39) from the largest Salvador sample produced solely female offspring (see Fig. 1). Likewise, the proportion of females among the female-biased broods from all other collections was above 98 % (15 in a total of 351 broods examined). It has been repeatedly shown that *Spiroplasma* are sensitive to physiological (e.g., mother's age), genetic, and environmental factors, which can affect the transmission and expression of male-killing [1, 3, 18, 26, 29, 32, 34]. Thus, our findings of high transmission rates and strong male-killer phenotype suggest the absence, or very low frequency, of transmission and expression modifiers in the sampled populations.

Non-Male-Killing *Spiroplasma*

The occurrence of non-male-killing *Spiroplasma* has been reported to occur in low frequency (under 1 %) in one *D. melanogaster* population in North America [44]. Similar, though higher, prevalences were found in the present study (3.0 and 5.0 %). It is worth noting that the non-male-killing *Spiroplasma* was found to have a lower prevalence than that of the male-killing in the population of Salvador in 2008 and was absent in Rio de Janeiro and Recife populations. Assuming that both have a high transmission rate and that they induce the same phenotypic side effects, it may be suggested that the difference in prevalence can be caused by the male-killing effect itself. If this is correct, the male-killer phenotype may favor the endosymbiont spread in populations through, for example, the resources reallocation from dead males to their female siblings, as previously proposed [20] but seldom tested [7, 27]. Watts et al. [44] argue that the male-killing effect does not appear to explain the presence of *Spiroplasma* bacteria in *Drosophila* species since the male-killing strains are restricted to some lineages, and the non-male-killing strains can reach high prevalence. This can be true considering the occurrence among species, but the results reported here suggest that within one population, the male-killing *Spiroplasma* may have a spreading advantage. This comparison is particularly relevant since the occurrence of both strains in the same population allows us to ignore environmental and genetic differences among populations (or species) and test the effects of the male-killing manipulation. In this context, further investigation is clearly required.

Spiroplasma and *Wolbachia* Association

When two vertically transmitted bacterial genotypes coexist for several generations, they are expected to evolve towards cooperation due to partner fidelity feedback [39]. This trend depends on their transmission efficiency; if bacteria have a high transmission rate, such as *Wolbachia* [16] and *Spiroplasma* [20] infecting *Drosophila*, the benefit of cooperation may not outweigh its costs [42]. No evidence of positive or negative association between *Wolbachia* and male-killing *Spiroplasma* infections was found in the present study, neither within a population nor across localities. Goto et al. [11] reported that male-killing *Spiroplasma* negatively affects *Wolbachia* density within *D. melanogaster* hosts. Based on the results described here, these interactions in an individual do not seem to affect the infection status at the population level. It is worth noting that Goto et al. [11] 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 artificial system studied by them. It is even possible that in the populations reported here, *Spiroplasma* and *Wolbachia* coevolved to a neutral association.

Phylogenetic Position of *Spiroplasma*

All the sequenced *Spiroplasma* are identical, without differences across the three localities. Likewise, they confirm the high similarity with the strains infecting *D. melanogaster* in Uganda, which suggests the infection's common origin [35]. Furthermore, the results reinforce that all the male-killing strains found so far in *Drosophila* are restricted to the *poulsonii* clade, whereas the non-male-killing strains are more diverse [13]. Additionally, because the non-male-killing strain reported here is genetically so similar to the male-killing one, it constitutes the ideal strain to be used in comparisons to investigate the mechanisms and possible outcomes derived from the male-killing ability.

Heterogeneity in *Spiroplasma* Prevalences

The results reported here offer the possibility to evaluate the temporal and spatial status of *Spiroplasma* infection in *D. melanogaster* populations. Considering time, there was an apparent decrease in *Spiroplasma* prevalence across the years for the Salvador population. Despite the high coefficient of determination found (R^2=0.98), the differences were not significant since only 3 years were evaluated, but there is a suggestive decrease. Future samples might confirm this hypothesis, but at least, there is no indication that the infection is spreading in this population.

The varying prevalence in different collection sites is also in agreement with previous *Drosophila* studies [44, 46]. Prevalence of male killers is determined by indirect effects of infection on female performance and the vertical transmission fidelity [17]. These parameters can be affected by many host and environmental conditions. We tested if temperature, *Wolbachia* infection, and major phylogenetic differences in the bacteria can explain the variation in the estimated prevalences of *Spiroplasma* among localities.

Briefly, we found (1) no correlation between the temperature of collecting localities and the estimated prevalences (although temperature is an important factor influencing transmission rates [1, 18, 29, 34]); (2) no positive or negative association between *Wolbachia* and *Spiroplasma* endosymbionts across localities; and (3) that *Spiroplasma* strains for the three localities are very closely related, suggesting the absence of large genetic differences among them (it is not impossible, however, that relevant genetic differences are present among populations).

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* [31, 35, 46] and from the *tripunctata* radiation of the subgenus *Drosophila* [32]. 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 conclusion, we found a large variability in the prevalences of male-killer *Spiroplasma* in Brazilian populations, but no evidence of any tendency for its increase in frequency. Furthermore, the measured variables are not sufficient to explain the spatial variation in the frequency of the endosymbiont. In this scenario, a combination of other host and environmental conditions, as well as population historic factors, may be responsible for establishing the heterogeneity reported in this study. This shows the difficulty in pinpointing the factors that actually govern the dynamics of inherited organisms in the field.

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Conflict of Interest The authors declare no conflict of interest.

Data Archiving Sequence data will be submitted to GenBank after the article acceptance, in accordance with the Instructions for Authors.

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Artigo 2

Low temperature reveals genetic variability against male-killing *Spiroplasma* in *Drosophila melanogaster* natural populations

Iuri M. Ventura, Thais da Costa, Louis B. Klaczko

Abstract

Spiroplasma endosymbionts are maternally inherited microorganisms which infect many arthropod species. In some *Drosophila* species, it acts as a reproductive manipulator, spreading in populations by killing the sons of infected mothers. Distinct *D. melanogaster* populations from Brazil exhibit variable male-killing *Spiroplasma* prevalences. In this study, we investigated the presence of genetic variability for the male-killing phenotype among *Drosophila* and/or *Spiroplasma* strains, and verified if it correlates with the endosymbiont prevalence in natural populations. For that, we analyzed the male-killing expression when *Spiroplasma* lineages from different populations were transferred to a standard *D. melanogaster* strain (Canton-S); and when a common *Spiroplasma* strain was transferred to different wild-caught *D. melanogaster* strains, both at optimal and challenging temperatures for the bacteria. No variation was observed in the male-killing phenotype induced by different *Spiroplasma* strains. No phenotypic variability among fly strains was detected at optimal temperature (23ºC) as well. Conversely, significant variation in the male-killing expression was observed among *D. melanogaster* strains at 18.5ºC, probably caused by imperfect transmission of the endosymbiont. Distinct strains differed in their average sex ratios, as well as in the pattern of male-killing expression as the infected females aged. Greater variation occurred among lineages from one locality, although there was no clear correlation between the male-killing intensity and the endosymbiont prevalence in each population. Imperfect transmission or male killing may also occur in the field, thus helping to explain the low or intermediate prevalences reported in nature. We discuss the implications of our results for the dynamics of male-killing *Spiroplasma* in natural populations.

Introduction

Endosymbiotic bacteria are maternally transmitted microorganisms which infect a large number of arthropod species (Duron *et al.*, 2008; Zug & Hammerstein, 2012). They have evolved the ability to induce several different manipulations of host's reproduction, including male killing, cytoplasmic incompatibility, parthenogenesis and feminization (Engelstadter & Hurst, 2009). Such manipulations are believed to contribute to the spread and maintenance of the endosymbionts in populations (Unckless & Jaenike, 2012). In the male-killing strategy, sons of infected mothers are killed by the bacteria, resulting in the production of female-biased broods. The death of males may benefit infected females by decreasing competition or inbreeding among siblings (Hurst & Majerus, 1993).

Bacteria of the genera *Spiroplasma* and *Wolbachia* were found to occur in several *Drosophila* species, at variable prevalences (Mateos *et al.*, 2006). In Brazilian natural populations, where *Spiroplasma* was first described in *D. melanogaster* (Montenegro *et al.*, 2000; 2005), it typically occurs at low frequencies (under 3% of females are infected), but higher prevalences were found in one locality (Salvador), where it reached around 17% (the highest estimate) (Montenegro *et al.*, 2005; Ventura *et al.*, 2012). *Wolbachia* has been found to be more common, infecting above 80% of *D. melanogaster* females in these localities. The prevalences reported for Brazil are in line with findings in other continents (Pool *et al.*, 2006; Watts *et al.*, 2009; and others summarized in Haselkorn, 2010).

The parameters governing the prevalence of male-killing endosymbiotic microorganisms are primarily the transmission efficiency from mother to offspring, the penetrance of the induced effect (male killing), and the fitness effects of infection on female hosts (this includes indirect consequences on infected individuals and possible advantages conferred by the male-killing manipulation itself) (Hurst & Jiggins, 2000; Engelstadter &

Hurst, 2009). Moreover, a number of factors can affect those parameters, such as environmental and host and/or bacteria constraints (Cavalcanti *et al.*, 1957; Hurst *et al.*, 2000; Kageyama *et al.*, 2009). In this context, temperature conditions have repeatedly been shown to play an important role in *Spiroplasma* transmission and reproductive manipulation. Generally, non-standard temperatures (low or high) decrease transmission rates (Montenegro & Klaczko, 2004; Anbutsu *et al.*, 2008; Osaka *et al.*, 2008). In addition, the existence of genetic factors in the hosts that affect the expression of reproductive parasites was demonstrated both between species (Poinsot *et al.*, 1998; McGraw *et al.*, 2002; Tinsley & Majerus, 2007; Jaenike, 2007b; Hutchence *et al*, 2012; Veneti *et al.*, 2012) and within strains of the same species (Yamada *et al*., 1985; Kageyama *et al.*, 2009).

It remains unclear, however, how those parameters actually interact to drive endosymbionts prevalence in natural populations (but see: Jaenike *et al.*, 2010 and Unckless & Jaenike, 2012). As mentioned above, *Spiroplasma* prevalence differs consistently among populations from distinct Brazilian localities, being low in Recife and Rio de Janeiro, and higher in Salvador (Ventura *et al.*, 2012). Besides infection distribution of male-killing *Spiroplasma*, several different issues have been addressed in these populations, such as the advantage of the male-killing strategy (Martins *et al.*, 2010); the indirect effects of infection (Montenegro *et al.*, 2006); the existence of non-male-killing *Spiroplasma* and the association with *Wolbachia* (Ventura *et al.*, 2012).

This is an opportune scenario to test the presence of genetic variability for the male-killing expression among *Drosophila* and/or *Spiroplasma* strains, and to verify if it correlates with the endosymbiont prevalence in natural populations. In this study, the intensity of male-killing was analyzed when *Spiroplasma* lineages infecting different populations were transferred to a standard *D. melanogaster* strain, and when a common *Spiroplasma* strain was

transferred to different wild-caught *D. melanogaster* lineages, both at optimal and challenging temperatures.

Methods

In this study we aimed to detect variability both among *Spiroplasma* and among *Drosophila* strains collected in different localities.

Strains

To assess the variability in *Spiroplasma* we used strains carried by different *Drosophila* isofemale lines derived from infected wild flies caught in different places, and on different occasions. There were seven bacterial donor lines (reported in Montenegro *et al.*, 2005; Ventura *et al.*, 2012 and Pool *et al.*, 2006): RED42 and RELH57 (collected in Recife, Pernambuco, 2004); RCH2 and NIA5 (from Rio de Janeiro, 2008 and 2010); SSB10 and SSJ38 (from Salvador, Bahia, 2008 and 2010); and UG-SR (from Namulonge, Uganda, 2005). These strains are routinely maintained in the laboratory by crossing them with uninfected Canton-S males (except UG-SR, which is crossed with UG-12 males, from the same locality).

The source of variability among *Drosophila* was provided by twelve isofemale lines collected in Rio de Janeiro (NIA-1, 2, 3, 8, 9, 10) and Salvador (SSJ-27, 37, 46, 48, 75, 81) in 2010. These lines have been kept isolated from one another in the laboratory. As mentioned above, populations from these localities exhibit consistent differences in the prevalence of *Spiroplasma*. All these lines are also infected with *Wolbachia*, providing the same background with which *Spiroplasma* interact in natural populations, where the prevalence of that endosymbiont is very high (Ventura *et al.*, 2012).

Screening for variability among *Spiroplasma* strains

In order to detect variation in the induction of male killing by the *Spiroplasma* strains collected in different localities, they were artificially injected into a standard fly strain and the sex ratios of the progenies were analyzed. Briefly, hemolymph from infected strains was microinjected in the thorax of Canton-S females, as described in Montenegro *et al.*, 2000. Once infected, the females (identified with the *Spiroplasma* donor strain) were separated in two groups and reared on cornmeal molasses medium, at 23ºC and 18.5ºC, for three generations, followed by PCR confirmation (detailed below). This procedure ensures that the *Spiroplasma* infection was well-established in each line, and that occasional variations in the male-killing phenotype were not an experimental artifact. Then, fifteen virgin females (F_1) were collected from each line and crossed with two Canton-S males, up to six hours after emergence. They were individually placed in 40 ml vials containing medium, and transferred to new vials every 2 days, for 10 days. All flies emerging from each vial in the five successive broods (F_2) were scored, and their sex ratios were examined. Only mothers that were confirmed to be infected by PCR and progenies from vials containing more than 12 flies were included in the analysis.

Screening for variability among *Drosophila* strains

To detect variation in the male-killing phenotype induced in different *D. melanogaster* strains, hemolymph from a single infected lineage (SSJ38, from Salvador) was transferred to the twelve strains mentioned earlier. Analysis of the offspring sex ratios was conducted as described above, except that the infected females were crossed with males from their own original lines; thus maintaining the same host genotype.

Confirmation of *Spiroplasma* infection

For both experiments, the *Spiroplasma* infection was confirmed by PCR in the mothers (F_1), as well as in a sample of ten individuals from each lineage. In order to investigate the cause of variation on male-killing effects, males that occasionally survived were individually tested for the endosymbiont infection (at least 10 males from each strain were tested). For that, after DNA extraction, PCR was performed using primers Spiro16S (Ventura *et al.*, 2012), with negative and positive controls (Canton-S and RED42 strains, respectively).

Analyses of sex ratios

These experiments allow describing the sex ratios trajectories in each infected line, following the increasing age of the mothers. We used the average proportion of females from each line (with up to fifteen replicates in each one) as the dependent variable in an ANCOVA, with strains as independent variable and the age of the mother as a covariate, with interaction. We used the mean of the replicates, instead of the replicate values, since the mean approaches normality even if the original data are not normally distributed (Zar, 1999).

This design is valuable for isolating endosymbiont and host genotypes effects, allowing separate testing. The comparison is improved with the sex ratios analysis in two different temperatures, an ideal for *Spiroplasma* transmission (23ºC) and a more challenging one (18.5ºC). Furthermore, the artificial transference of *Spiroplasma* ensures that it interacts with the same host genetic background found in natural populations (including mitochondrial genome).

Results

Variability among *Spiroplasma* strains

Sexual proportions induced in Canton-S line by distinct *Spiroplasma* strains showed very little variability. All strains tested induced a strong male-killing phenotype, both at 18.5ºC and 23ºC. In the higher temperature, all replicates from all lines originated solely females (N = 12 063 flies were scored); whereas at the lower temperature, very few males appeared in only two lines (NIA5 and SSB10). However, the sexual proportions were again strongly biased (average sex ratios in these two lines were 97.8% and 98.8%, respectively). Furthermore, only two replicates in each line presented some males. Therefore, the small difference among strains were not significant (N = 9 141).

Variability among *Drosophila* strains

At 23ºC, all *Drosophila* strains exhibited complete male-killing phenotype, similar to the results described above (N = 26 908), without the emergence of a single male. At 18.5ºC, however, the survivorship of males was heterogeneous among strains (N = 15 278). Figure 1 summarizes the variations in the average proportion of females among the strains tested, in relation to the maternal age. Each line represents the average of up to fifteen replicates. Data from days 1-2 were not included in the analyses, because very few individuals emerged at 18.5ºC.

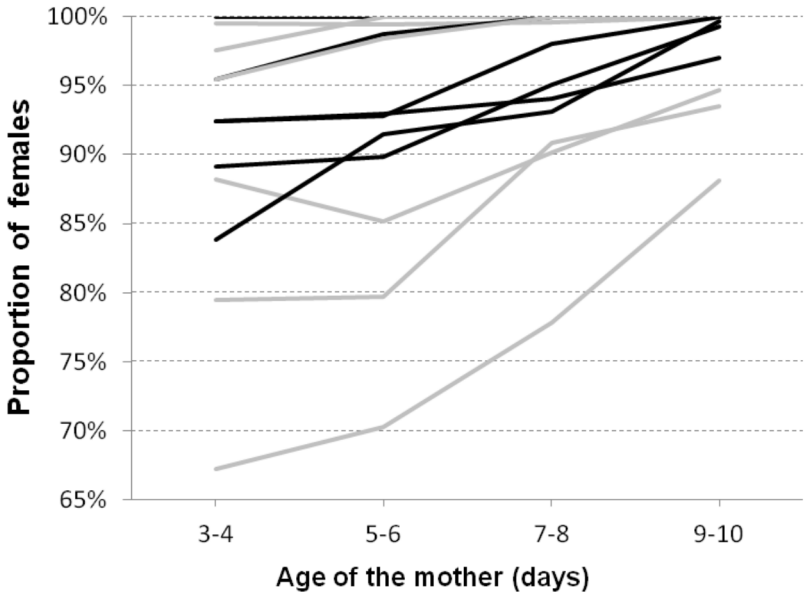

Figure 1 Trajectory of average proportion of females in each strain of *D. melanogaster*, in relation to the mother's age (days after emergence) at 18.5ºC. Each line represents the average of up to fifteen replicates. Gray lines correspond to fly strains collected in Salvador and black lines, those collected in Rio de Janeiro.

The effects of strains, maternal age and their interaction were tested with an ANCOVA. It demonstrated that sexual proportions varied significantly according to the age of the mothers ($F = 133.6$; d.f. = 1; $p < 0.001$), as well as according to strains ($F = 11.4$; d.f. = 11; $p < 0.001$). Furthermore, the interaction between strain and maternal age had a significant effect on the determination of sex ratios ($F = 3.1$; d.f. = 11; $p < 0.01$).

Then, the strains were grouped according to their origins. Average proportions of females among the Salvador and Rio de Janeiro strains were, respectively, 91.4% and 95.6%. This time, locality had no significant effect (ANCOVA, $F = 2.01$; d.f. = 1; $p > 0.16$). Nevertheless, when the lines' sex ratios were grouped by locality, a significant variation between their variances was demonstrated (variances for Salvador and Rio de Janeiro lines were, respectively, 0.040 and 0.018; F-test; $F = 2.24$; d.f. = 23, 23; $p < 0.05$).

Lastly, PCR assay evidenced that all males which appeared in the variability experiment among *Drosophila* strains were not infected with *Spiroplasma*. Totally, ninety males from six strains were tested. In addition, all lineages used in the experiment were confirmed to be infected with *Spiroplasma* (N = 120 females were tested).

Discussion

The transmission from mothers to progeny is essential for endosymbiotic bacteria to persist and spread in populations (Hurst & Jiggins, 2000). Two other determining factors for the prevalence of selfish bacteria, such as *Spiroplasma*, are: the intensity of reproductive manipulation of the hosts and the indirect effects on infected individuals (Engelstadter & Hurst, 2009). On the other hand, it is expected that natural selection favors resistance modifiers in the host species (Jaenike, 2007a). However, it is not easy to correlate variations in these factors with actual prevalences in natural populations (but see: Jaenike *et al.*, 2010; Unckless & Jaenike, 2012 for recent attempts).

The screening among *Spiroplasma* strains described here suggests that there is little or no variation for the male-killing ability in the lines infecting *D. melanogaster* in populations from Brazil and Uganda. All seven lines induced strong male killing in Canton-S flies, both at low and high temperatures. This result matches the report by Kageyama *et al.* (2009), which also describes high penetrance of male-killing manipulation in Canton-S. Furthermore, the lack of phenotypic variation among the *Spiroplasma* strains is compatible with the lack of genetic variation observed among them in two genes (16S rRNA and *fruR*; Ventura *et al.*, 2012). Therefore, despite the differences of prevalence in the field, *Spiroplasma* bacteria found in distinct localities probably induce similar effects and are genetically very close. This suggests a common infection origin in *D. melanogaster* (Pool *et al.*, 2006).

Screening among fly strains from Salvador and Rio de Janeiro detected no variability at 23ºC, with the induction of complete male killing in all strains tested. These findings are consistent with data gathered from several summer collections in natural populations, which showed very low frequencies of males in more than 50 progenies infected with *Spiroplasma* (Ventura *et al.*, 2012).

Conversely, screening among fly strains exhibited interesting variation at 18.5ºC. A considerable proportion of males appeared, especially in progenies from younger mothers, and suggested heterogeneity among strains (Figure 1). ANCOVA results demonstrated that sex ratios vary significantly depending on the age of the mothers, as well as the strains infected. The first correlation (with maternal age) is commonly observed in experimental studies using *Spiroplasma* (Kageyama *et al*, 2009; Hutchence *et al.*, 2012) and it is probably related to bacterial density, which increases transmission efficiency in older females (Anbutsu & Fukatsu, 2003). Considering all strains at 18.5ºC, average proportion of females increased from 90.0% in the days 3-4 to 97.7% in the days 9-10.

Sex ratios also varied among the distinct strains tested. Likewise, for the male-killing phenotype penetrance, a significant interaction was detected between strains and maternal age. That is, distinct strains not only differed in their average sexual proportions, but also in their trajectories along the days (i.e., the line slopes in Figure 1). This suggests that genetic backgrounds from distinct strains interact differently with the *Spiroplasma* endosymbiont, resulting in different penetrances of the male-killing phenotype. But this variability is only revealed at 18.5ºC, a harsher temperature for *Spiroplasma* transmission.

Similar effects of host background against *Spiroplasma* manipulation were already reported in *D. melanogaster* natural and standard laboratory strains (Yamada *et al*., 1985; Kageyama *et al.*, 2009, respectively). Our data also showed the delay of male-killing

expression in some strains under relatively low temperatures, rather than complete resistance of the males.

Three hypotheses could be raised to explain the survivorship of sons from infected mothers: resistance to male-killing induction; low density of bacteria in the sons; or failure of bacterial vertical transmission. The last hypothesis is more plausible to explain the results reported here, since PCR essays failed to detect the presence of *Spiroplasma* in all surviving males tested. Therefore, the retardation of male-killing expression may occur when the endosymbiont density in females lays below the threshold for perfect transmission and death of all males (Anbutsu & Fukatsu, 2003); whilst complete resistance might require variation in highly conserved developmental pathways, with which *Spiroplasma* interact (Veneti *et al.*, 2005). This would explain the reiterated findings of subtle variation of male-killing expression in *Drosophila*.

Our data provide the rare opportunity to try to correlate experimental results with prevalences in natural populations. Different collections have shown that *Spiroplasma* infection occurs at medium frequencies in Salvador (9.6 – 17.7%); while they have lower frequencies in other localities (at most 2.3% in Recife, or 1.7% in Rio de Janeiro) and this difference does not seem to be correlated with average temperature (Ventura *et al.*, 2012). Our results did not detect a clear correlation between the intensity of male-killing expression and the origin of the *Drosophila* strains tested. That is, the intensity of the sexual manipulation induced by *Spiroplasma* in the Salvador lines does not explain the higher prevalence reached at this locality, as could be suggested. Nevertheless, we found that distinct populations showed different levels of genetic variation in the expression of the male-killing phenotype. This is supported by the observation that there is greater variability in the sex ratios among the Salvador lines than in the Rio de Janeiro ones (see Figure 1).

Therefore, the scenario of *Spiroplasma* distribution in these populations could be described as follows: considerable difference of the endosymbiont prevalence is not accompanied by phylogenetic or phenotypic variation among *Spiroplasma* strains. No phenotypic variation occurs among fly strains at optimal temperature (23ºC) as well. However, if perfect transmission occurred in the field (as described here at 23ºC), *Spiroplasma* prevalence should be much higher (or at least increasing) in the populations, which was not observed (Ventura *et al.*, 2012). Therefore, imperfect transmission (such as those reported here for 18.5ºC) or other factors (such as detrimental side-effects of infection) must act to maintain the prevalence at low or intermediate levels.

Concluding, variation among strains of *D. melanogaster* in male-killing susceptibility was shown to occur, depending on the temperature and the age of the infected females. Therefore, *Spiroplasma* reproductive manipulation and transmission in non-optimal temperatures, combined with environmental variation, may explain part of the prevalence differences in the field. This hinders our ability to accurately predict prevalences and the fate of an endosymbiont infection in natural populations using simplistic models. In this context, realistic models should incorporate variations in host and environmental parameters to describe the population dynamics trends of endosymbionts.

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Conclusões

- A prevalência de *Spiroplasma* androcida variou de 0 a 17,7% (a mais alta estimativa encontrada para uma espécie de *Drosophila*). A prevalência nas populações de Recife e Rio de Janeiro são significativamente inferiores à de Salvador.

- Também foi observada a ocorrência de *Spiroplasma* não androcida na população de Salvador, em prevalência inferior ao da linhagem androcida (3,0 – 5,0%), o que pode sugerir uma vantagem de disseminação da estratégia androcida.

- Todas as linhagens do endossimbionte analisadas agrupam-se proximamente no clado de *Spiroplasma poulsonii*.

- A prevalência de *Wolbachia* foi estimada entre 81,8 – 100% nas populações amostradas. Não detectamos associação entre as prevalências de *Spiroplasma* e *Wolbachia* dentro de nossa maior amostra, nem entre as populações amostradas.

- Não foi observada variação no fenótipo androcida induzido por diferentes linhagens de *Spiroplasma* em uma linhagem padrão de *Drosophila* em duas temperaturas experimentais (18,5ºC e 23ºC).

- Por outro lado, foi observada variação significativa no fenótipo androcida de diferentes linhagens de *Drosophila* na temperatura mais baixa (18,5ºC), provavelmente causada por falhas na transmissão da bactéria.

- Não há uma relação clara entre a intensidade do efeito androcida induzido nas linhagens e a prevalência estimada em cada localidade. Porém, uma variação maior foi observada entre linhagens da localidade de Salvador, em comparação com as do Rio de Janeiro.

- Falhas na transmissão e no efeito androcida podem ajudar a explicar as prevalências baixas e médias observadas para *Spiroplasma* em populações naturais de *D. melanogaster*.