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"Sex-Ratio" Citoplasmático em *Drosophila melanogaster*

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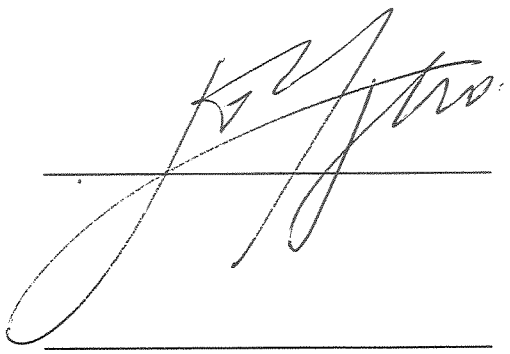
Prof. Dr. Louis Bernard Klaczko (Orientador) 

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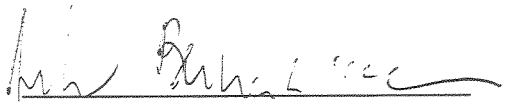
Prof. Dr. Antonio Bernardo de Carvallho 

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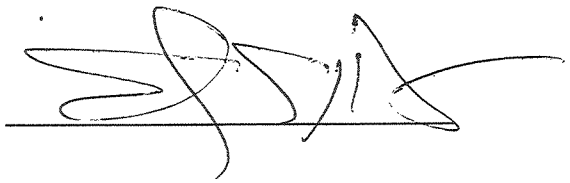
Prof. Dr. Gonçalo Amarante Guimarães Pereira 

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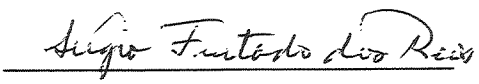
Prof. Dr. Sérgio Furtado dos Reis 

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"A sorrir

Pretendo levar a vida,

Pois chorando

Eu vi a mocidade perdida"

Cartola

Índice

Resumo

Elementos genéticos egoístas são partículas herdáveis de etiologia e mecanismos de ação variados, mas com a caracterítica em comum de modificar a segregação mendeliana dos alelos em favor próprio, aumentando a própria freqüência. Elementos citoplásmaticos egoístas são um caso especial de elementos genéticos egoístas, como são herdados apenas pelo citoplasma materno, eles sofrem seleção para causar desvios na proporção sexual, em favor de um excesso de fêmeas.

A proporção sexual nas progênies de organismos que se reproduzem sexualmente é, em geral, 1♀: 1♂. Fisher (1930) propôs a primeira explicação baseada em seleção natural para esta proporção. O princípio de Fisher prediz que o sexo mais raro tem maior valor adaptativo, pois sua contribuição média por indivíduo é maior. Assim, a seleção favorecerá alelos que aumentem a freqüência do sexo raro nas progênies. Quando a proporção chegar a 1♀: 1♂, indivíduos dos dois sexos terão o mesmo valor adaptativo. Parasitas reprodutivos, endosimbiontes herdados maternalmete, representam a maior parte dos elementos citoplasmáticos egoístas, e uma das causas mais comuns de desvios à proporção de 1♀: 1♂.

Agentes androcidas, endossimbiontes que matam os embriões machos, são um destes parasitas reprodutivos. Como conseqüência, a proporção sexual da prole das fêmeas infectadas afasta-se de 1♀: 1♂, apresentando excesso ou totalidade de fêmeas. Agentes androcidas foram encontrados em mais de 40 espécies de 6 ordens de insetos. Várions táxons de bactérias já foram associados com o fenótipo, e a filogenia dos parasitas e hospedeiros não é congruente, indicando transmissão horizontal freqüente.

Neste trabalho, estudamos o agente androcida de *Drosophila melanogaster*, descrito recentemente. Sistemática molecular mostrou que trata-se de uma bactéria do gênero

Spiroplasma, muito próxima ao agente androcida encontrado em *D. nebulosa*. As seqüências comparadas (3000 pares de base, representando seqüências parciais de 3 genes) são idênticas, portanto, as duas bactérias têm um ancestral comum muito recente. De fato, possivelmente as duas espécies de *Drosophila* só entraram em contato há 4 ou 5 séculos, com a expansão mundial de *D. melanogaster*, e a transmissão do *Spiroplasma* de *D. nebulosa* para *D. melanogaster* deve ter ocorrido depois disso. Em uma coleta em Recife, foi encontrado que 2,3% das fêmeas estavam infectadas com *Spiroplasma*; outro parasita reprodutivo, a bactéria *Wolbachia* (que causa incompatibilidade citoplasmática) infectava 96% das fêmeas. Além disso, as duas bactérias co-infectavam os mesmos indivíduos, uma observação até agora incomum para parasitas reprodutivos com fenótipos diferentes.

Como parasitas reprodutivos tem transmissão exclusivamente materna, seu valor adaptativo é altamente correlacionado com o das fêmeas hospedeiras. Desta forma, espera-se que parasitas reprodutivos não tenham efeitos deletérios no valor adaptativo das fêmeas hospedeiras, ou até que sejam benéficos. Por outro lado, a eficiência de transmissão ou a intensidade do fenótipo de manipulação reprodutiva podem estar positivamente correlacionados com a densidade de bactérias no hospedeiro, de forma que parasitas mais eficientes causem efeitos deletérios no valor adaptativo da fêmea. Para verificar se existem efeitos de *Spiroplasma* e *Wolbachia* no valor adaptativo de fêmeas, medimos a viabilidade larval e a fecundidade de fêmeas adultas, comparando com a de fêmeas não-infectadas. Nenhuma das duas características parece ser afetada por *Spiroplasma* ou *Wolbachia*, nem por infecção com as duas bactérias simultaneamente.

Finalmente, foi verificado que temperaturas baixas (em torno de 16,5°C) interrompem a transmissão do agente androcida para a progênie das moscas infectadas.

Summary

Selfish genetic elements are inheritable particles with diverse ethiology and mechanisms, but sharing the fact that they modify the Mendelian segregation for their own benefit, thus increasing their own frequency. Selfish cytoplasmic elements are a special instance of selfish genetic elements, as they are inherited only by maternal cytoplasm, they suffer strong selection to alter the sexual proportion, in direction to an excess of females.

The usual sexual proportion in sexually reproducing organisms is 1♀: 1♂. Fisher (1930) was the first to propose an explanation based on the natural selection for this proportion. Fisher's principle predicts that, if one of the sexes is rarer, it will have higher fitness, because its individual average contribution will be larger. Thereafter, selection will favour alleles that increase the frequencies of the rarer sex in broods. When the proportion reaches 1♀: 1♂, both sexes will have the same fitness. Reproductive parasites, maternally inherited endosymbionts, are the majority of selfish cytoplasmic elements, and one of the most common causes of departures from the 1♀: 1♂ sexual proportion.

Male killers, endosymbionts that kill male embryos, are one of these reproductive parasites. Consequently, the sexual proportion of infected females deviates from 1♀: 1♂, towards an excess or totality of females. Male killers have been found in more than 40 species of 6 insect orders. Several bacterial taxa have been associated with male-killing phenotype, and the phylogeny of parasites ad hosts is not congruent, indicating frequent horizontal transmission.

In this work, we studied the recently described male killer agent found in *Drosophila melanogaster*. Molecular systematics showed that this agent is a *Spiroplasma* bacterium, closely related to the *D. nebulosa* male-killer. The compared sequences (3000 base pairs, partial sequence from 3 genes) are identical, thus both bacteria share a very recent common ancestral.

Indeed, only since *D. melanogaster* worldwide expansion, 4 or 5 centuries ago, both *Drosophila* species overlap in their distribution, and the *Spiroplasma* transmission from *D. nebulosa* to *D. melanogaster* probably occurred after this. In one collection in Recife, *Spiroplasma* has been found infecting 2.3% of all females, another reproductive parasite, the bacterium *Wolbachia* (which causes cytoplasmic incompatibility) infected 96% of all females. *Wolbachia* was also found co-infecting flies alongside with *Spiroplasma*, a rather uncommon observation for reproductive parasites with different phenotypes.

As reproductive parasites have exclusively maternal transmission, their fitness is highly correlated with its female host fitness. Consequently, it is expected that reproductive parasites do not pose deleterious fitness effects to their female hosts, or even be beneficial. However, transmission efficiency or the intensity of the reproductive manipulation phenotype could be positively correlated to the bacterial load on the host, in a way that more efficient parasites will have a deleterious fitness effect on females. In order to verify if *Spiroplasma* and *Wolbachia* have any kind of fitness effects on female hosts, we assessed larval viability and fecundity of adult females, in relation to uninfected females. Both characteristics showed signs of being affected by *Spiroplasma* or *Wolbachia*, nor by double-infection with both bacteria.

Finally, low temperature (approximately 16.5°C) interrupts transmission of the male killing agent to the progeny of infected females.

1) Introdução

1.1) Elementos genéticos egoístas

Elementos genéticos egoístas (**EGE**) são partículas herdáveis que produzem desvios nas proporções mendelianas, aumentando sua própria freqüência e geralmente prejudicando seu hospedeiro (Orgel & Crick, 1980; Hickey, 1982; Doolittle & Sapienza, 1980; Crow, 1988; Werren *et al.*, 1988). Os **EGE** são descobertos freqüentemente por acaso, devido a mudanças na razão sexual, taxas de mutação ou padrões de mortalidade. Sua etiologia, mecanismos e efeitos fenotípicos são variados. Existem genes que alteram a segregação meiótica (ou genes de impulso meiótico), tanto autossômicos – por exemplo, "segregation distorter" em *Drosophila melanogaster* (Hartl & Hartung, 1975) – como ligados aos cromossomos sexuais – presentes em muitas espécies de *Drosophila*, como *D. mediopunctata* (Carvalho *et al.*, 1989), *D. quinaria* e *D. recens* (Jaenike, 1996). Outros exemplos de **EGE** são os transposons (Kidwell, 1992), que fazem cópias de si mesmos em vários locais no genoma do hospedeiro; os genes letais de efeito materno *Medea* (Beeman & Friesen, 1999; Smith, 1998), que matam a prole que não possui uma cópia (materna ou paterna) do próprio gene; cromossomos B, cromossomos extras que podem se replicar independentemente dos outros cromossomos (Riera *et al.*, 2004); e finalmente "homing endonucleases genes", genes que produzem uma enzima que reconhece e corta cromossomos que não contêm uma cópia do gene, e o próprio sistema de reparo cromossômico do hospedeiro repara o dano, mas usando como molde o cromossomo contendo o gene (Goddard *et al.*, 2001; Posey *et al.*, 2004).

1.2) Elementos citoplasmáticos egoístas e parasitas reprodutivos

Elementos citoplasmáticos egoístas (**ECE**) representam um caso especial de **EGE** (Eberhard, 1980; Cosmides & Tooby, 1981). A assimetria entre transmissão biparental para os genes nucleares e exclusivamente materna para os genes citoplasmáticos é uma forte fonte de conflito. Como apenas as fêmeas transmitem os **ECE**, estes sofrem forte seleção para desviar a proporção sexual ou a alocação de recursos em favor das fêmeas. Alguns exemplos de **ECE** são: esterilidade citoplasmática do macho em plantas, causada por mutações mitoncondriais (Saumitou-Laprade *et al.*, 1994), incompatibilidade citoplasmática, causada pela bactéria *Wolbachia* (Hoffmann *et al.*, 1998) e muitos outros endossimbiontes que alteram a proporção sexual de 1♀: 1♂, como agentes androcidas (**AA**), que matam os machos antes destes atingirem a idade adulta (Williamson & Poulson, 1979; Hurst *et al.*, 1996a). A maior parte dos **ECE** é composta por bactérias ou outros microorganismos; apesar de terem origem filogenética variada, todos apresentam semelhanças quanto à dinâmica populacional e, potencialmente, quanto aos efeitos evolutivos nos hospedeiros. Por este motivo, eles são classificados como parasitas reprodutivos (Bandi *et al.*, 2001; O'Neill *et al.*, 1997).

Apenas dois exemplos de organelas egoístas foram descritos: a esterilidade citoplasmática do macho, causada por mitocôndrias em plantas (Saumitou-Laprade *et al.*, 1994) e os mutantes "petite" em *Saccharomyces cerevisiae* (MacAlpine *et al.*, 2001); não há nenhum exemplo descrito de alteração reprodutiva causado por cloroplasto. Por que não foram encontrados mais exemplos de mitocôndrias e cloroplastos egoístas? Existem duas explicações para isto:

1) o genoma destes organelas é pequeno, com relativamente poucos genes, e voltados para uma função específica. Assim, não há predisposição genética para que as organelas sejam manipuladoras.

2) As organelas são fundamentais na fisiologia dos organismos. Se uma organela egoísta se espalhar, ela pode levar a população à extinção, por acúmulo de efeitos deletérios ou por falta de machos.

1.3) Proporção sexual de Fisher

A proporção sexual de 1♀: 1♂ é a mais comumente encontrada na natureza, em organismos que se reproduzem sexualmente. Por ter sido Fisher o primeiro a propor uma explicação baseada em seleção natural (Fisher, 1930, para ver uma discussão sobre a precedência de Fisher, ver Edwards, 2000), ela é conhecida como proporção sexual de Fisher. O princípio de Fisher pode ser interpretado da seguinte maneira: em uma população reproduzindo-se sexualmente, metade dos genes vem de cada sexo, independente da freqüência dos sexos. Se algum sexo for mais raro, os indíviduos deste sexo terão valor adaptativo maior, pois sua contribuição, em média, será maior. Desta forma, a seleção favorecerá os alelos que aumentem a freqüência do sexo mais raro nas progênies. Quando a proporção chegar a 1♀: 1♂, não fará diferença investir preferencialmente em qualquer sexo, pois a seleção é dependente da freqüência – quanto maior o desvio para um lado, maior a pressão de seleção para o outro. Na verdade, o argumento anterior admite custos iguais entre a produção de um macho ou de uma fêmea. Caso isto não seja verdade, a seleção ocorrerá no sentido de equiparar o investimento entre os sexos.

Alguns pressupostos do princípio de Fisher (postulados pelo próprio Fisher ou por outros autores; revisão em Bull & Charnov, 1988) são:

1. sexos separados.
2. biparentalismo – todo zigoto tem uma mãe e um pai.
3. segregação mendeliana dos alelos que influenciam a proporção sexual.

4. controle parental da proporção sexual – o genótipo de um indivíduo influencia a razão sexual de sua progênie.

5. os custos da progênie são aditivos e os recursos para produzí-la, limitados.

6. população de tamanho infinito e panmítica.

7. as razões sexuais diferentes entre famílias não são correlacionadas com diferenças no valor adaptativo de um sexo.

Se algum destes pressupostos é quebrado, a proporção sexual poderá desviar-se de 1♀: 1♂. Por exemplo, em condições em que machos aparentados competem entre si por fêmeas aparentadas (violação do pressuposto 6), a razão sexual desvia-se para um excesso de fêmeas e a freqüência final de cada sexo dependerá da intensidade da competição por acasalamento entre os machos (Hamilton, 1967).

1.4) EGE, ECE e desvios na proporção sexual

EGE e **ECE** são uma fonte de alteração na proporção sexual de Fisher, pois violam pressupostos da teoria (Bull & Charnov, 1988). Genes de impulso meiótico (segregação não mendeliana) e partículas citoplasmáticas herdáveis (segregação não mendeliana e uniparentalismo) podem desviar a proporção esperada para até 100% de machos ou fêmeas, levando a população à extinção. De fato, progênies de algumas fêmeas coletadas na natureza apresentam total falta de machos – uma característica chamada de "sex-ratio" (**SR**). Os primeiros casos de **SR** analisados – *Drosophila obscura* (Gershenson, 1928) e *D. pseudoobscura* (Sturtevant & Dobzhansky, 1936) – eram determinados pelo genótipo paterno e causados por um cromossomo X que provocava impulso meiótico. Posteriormente foram descritos **SR** herdados maternalmente em diversas espécies de *Drosophila* (Buzzatti-Traverso, 1941; Cavalcanti *et al.*, 1957; e outros, revisão em Hurst & Jiggins, 2000). Todos apresentavam herança citoplasmática e

eram causados por agentes androcidas, que agem no estágio de embrião e, em algumas ocorrências, demonstrou-se que os **AA** eram infecciosos (Malogolowkin & Poulson, 1957).

Howard (1942) deparou-se com um exemplo intrigante e complexo no tatuzinho de jardim *Armadillidium vulgare*, com progênies com excesso ou inteiramente de machos, com excesso ou inteiramente de fêmeas, além de progênies com razão sexual normal. Ele interpretou o fenômeno como resultate de impulso meiótico dos cromossomos sexuais nas fêmeas, tanto do Z como do W, produzindo proles com excesso de machos e/ou fêmeas. Mais recentemente, Juchault *et al.* (1992) explicaram a ocorrência como devida a dois fatores **SR**, um citoplasmático – uma bactéria do gênero *Wolbachia* – e o outro, de origem desconhecida, com transmissão predominantemente (mas não exclusivamente) materna. Os dois fatores agem como indutores de feminização (**IF**), e um gene nuclear dominante inibe ambos. Aliás, Cavalcanti *et al.* (1958) já chamavam a atenção para o fato que a ação simultânea de fatores citoplasmáticos e modificadores nucleares pode produzir resultados complexos, complicando a interpretação de resultados: apenas depois de obter diversas estirpes homozigotas em *D. prosaltans* foi possível encontrar padrões previsíveis e descrever o **SR** observado como resultado de um agente androcida e um gene nuclear recessivo que interrompe a transmissão do **AA**. Finalmente, indutores de partenogênese (**IP**) nas vespas parasitóides *Trichogramma pretiosum* e *T. deion* também provocam proles com fêmeas exclusivamente (Stouthamer & Werren, 1993).

1.5) Agentes androcidas

Fêmeas infectadas por indutores de feminização, indutores de partenogênese ou agentes androcidas têm a prole com excesso de fêmeas, comumente apresentando total falta de machos. Os três elementos são citoplasmáticos e transmitidos apenas por fêmeas. Quando estes elementos encontram-se em equilíbrio numa população, a proporção de fêmeas infectadas produzidas numa

geração deve ser igual à proporção de fêmeas infectadas da geração anterior, em relação ao tamanho total da população (ou $Ni_t/Nf_t=Ni_{t-1}/Nf_{t-1}$, onde Ni representa o número de fêmeas infectadas, Nf o total de fêmeas da população e t a geração). Como fêmeas normais podem ser filhas de fêmeas normais ou de fêmeas infectadas (quando a transmissão for menor do que 100%), e fêmeas infectadas só podem ser filhas de fêmeas infectadas, a prole (feminina) de fêmeas infectadas deve ser maior do que a prole (feminina) de fêmeas não infectadas, para compensar a transmissão incompleta. Como **IP** e **IF** são **ECE** que transformam toda "prole potencial" (fêmeas e machos) da fêmea infectada em fêmeas, a progênie de uma fêmea infectada tem o dobro de fêmeas comparada com a progênie de uma fêmea normal. Portanto, para **IP** e **IF** as condições de invasão são pouco restritivas, e espera-se que a freqüência de equilíbrio seja alta – mais do que 50% das fêmeas infectadas – e que a proporção sexual da população apresente um desvio acentuado para excesso de fêmeas (Hurst, 1993), apenas em caso de transmissão muito ineficiente ou de efeitos deletérios muitos grandes estes **ECE** não irão persistir na população.

Agentes androcidas, ao contrário, matam metade dos embriões produzidos pelas fêmeas infectadas (os machos). Mesmo com transmissão totalmente eficiente e sem efeitos deletérios no valor adaptativo, o número de fêmeas das proles de fêmeas infectadas é igual ao número de fêmeas de proles de fêmeas normais. Desta forma, as condições de invasão de um **AA** são bastante restritivas: apenas nos casos em que a mortalidade dos machos confere alguma vantagem para as fêmeas infectadas sobreviventes, ou se houver transmissão horizontal, existe a chance de o **AA** manter-se população (Hurst, 1991).

Hurst (1991) propôs a separação entre **AA** precoces (agem no começo da embriogênese) e **AA** tardios (agem no final da fase de larva), argumentando que a lógica evolutiva subjacente aos dois fenômenos é completamente diferente. **AA** precoces são favorecidos por seleção de parentesco. Parentes clonais do elemento suicida localizados nas fêmeas beneficiam-se

indiretamente com a morte dos machos, pelo aumento do valor adaptativo destas fêmeas. Este aumento do valor adaptativo pode ser devido à diminuição da competição entre a prole restante, ou devido à diminuição da depressão por endocruzamento (Werren, 1987; Hurst, 1991). A morte dos machos acontece cedo (antes da eclosão dos ovos) aumentando ao máximo o benefício para as fêmeas sobreviventes. Em **AA** tardios, a morte dos machos libera uma grande quantidade de **AA**, que infectam um hospedeiro intermediário. Depois, há transmissão do hospedeiro intermediário para fêmeas não infectadas (Hurst, 1991). A morte dos machos acontece tarde (no último instar larval) depois de o agente androcida ter se reproduzido até alcançar alta densidade dentro do hospedeiro definitivo, e liberar um grande número de parasitas para aumentar a eficiência de transmissão para o hospedeiro intermediário.

1.6) Diversidade de parasitas reprodutivos

Apesar das condições relativamente restritivas para invasão, **AA** precoces parecem ser um fenômeno comum entre artrópodes, ocorrendo em várias espécies de diferentes níveis taxonômicos. Em insetos, já foram descritos em mais de 40 espécies de 5 ordens (Hurst 1991; Ebbert, 1991; Hurst & Jiggins, 2000), com freqüências extremamente variáveis, de 1% (Williamson & Poulson, 1979) a 99% (Dyson & Hurst, 2004). Em *Drosophila*, **AA** precoces foram relatados em dez espécies: *D. bifasciata* (Buzzatti-Traverso, 1941; Magni, 1953; Moriwaki & Kitagawa, 1954), *D. prosaltans* (Cavalcanti *et al.*, 1957), *D. robusta* (Poulson, 1966), *D. borealis* (Carson, 1956), *D. willistoni* e *D. paulistorum* (Malogolowkin, 1958), *D. equinoxialis* (Malogolowkin, 1959), *D. nebulosa* (Poulson, 1963), *D. mercatorum* (Barros, 1949) e *D. roehrae* (Vaz *et al.*, 1998).

Até agora, cinco taxa de bactérias foram associados a mortalidade precoce de machos em insetos, e dentro de dois destes taxa parece ter havido mais de uma evolução independente. (Hurst

& Jiggins, 2000; Hurst *et al.*, 2003; O'Neill *et al.*, 1997). Esta diversidade é o oposto do observado para as outras manipulações reprodutivas, nas quais *Wolbachia* é quase que exclusivamente a única bactéria causadora. Com base nos casos em que o **AA** foi identificado, Hurst *et al.* (2003) propuseram que, provavelmente, a diversidade de bactérias causadoras não é muito maior do que a conhecida até o presente. No entanto, a maior parte dos casos estudados está concentrada em alguns taxa de insetos (lepidópteros, coleópteros e drosofilideos), e a descoberta de agentes androcidas em outros taxa pode revelar ainda outros agente androcidas.

Recentemente, técnicas de genética molecular têm sido usadas na caracterização e identificação de bactérias, com enorme sucesso, principalmente pela possibilidade de trabalhar sem a necessidade de se cultivar a linhagem bacteriana (Amann *et al.*, 1995). Basicamente, no caso de **AA** a estratégia consiste em extrair o DNA de ovários ou de ovos do hospedeiro, e realizar reações de amplificação de DNA através de PCR, usando *primers* específicos para bactérias. O produto obtido pode ser seqüenciado diretamente ou após clonagem. Com a seqüência, identifica-se a bactéria e desenham-se ou escolhem-se na bibliografia *primers* específicos para o grupo identificado, para testar a associação entre **AA** e bactéria. Vários **AA** foram identificados com esta metodologia, e apenas bactérias foram identificadas como causadoras de **AA** precoces até agora (Hurst & Jiggins, 2000).

1.7) Evolução de parasitas reprodutivos

Os modelos clássicos de virulência de parasitas predizem que parasitas com transmissão exclusivamente vertical evoluirão para redução da virulência e, eventualmente, para uma relação mutualista com o hospedeiro; por outro lado, parasitas com transmissão horizontal evoluirão para aumento da virulência (Fine, 1975; Ewald, 1987; Yamamura, 1993; Lipsitch *et al.*, 1995). No entanto, o fato de existirem formas alternativas de o parasita aumentar sua freqüência faz com

que as conclusões destes modelos sejam incompletas (Toft & Karter, 1990; O'Neill *et al.*, 1997). Existem vários níveis em que a seleção pode atuar nos parasitas reprodutivos, por exemplo, dentro dos hospedeiros – onde a seleção natural vai favorecer parasitas mais virulentos, se a virulência estiver correlacionada com transmissão vertical – e entre hospedeiros diferentes – onde a seleção natural será a favor dos parasitas menos virulentos. Assim, estimar os parâmetros da interação entre parasita e hospedeiro é fundamental, tanto para prever a dinâmica populacional entre hospedeiro e parasita, como para especular sobre a evolução da simbiose (Dunn *et al.*, 1995). Por exemplo, Turelli (1994), modelando incompatibilidade citoplasmática para *Drosophila simulans/Wolbachia*, mostra que a seleção natural poderá favorecer parasitas menos virulentos, se variantes menos virulentas tiverem a mesma eficiência de transmissão e resgatarem a modificação de incompatibilidade citoplasmática causada pelas variantes mais virulentas.

1.8) "Sex-ratio" e *Drosophila melanogaster*

Drosophila melanogaster é uma das espécies mais estudadas em biologia, sendo um modelo em genética (Rubin, 1988; Powell, 1995). É uma espécie cosmopolita, de distribuição mundial e, até recentemente, não havia nenhum registro de um endossimbionte androcida em populações desta espécie (Fitz-Earle & Sakaguchi, 1986; Hurst, 1993). Em 1997, coletando em mercados de Campinas, por acidentente encontramos uma fêmea que apresentou progênie composta exclusivamente de fêmeas. Inicialmente, o estoque foi mantido cruzando as fêmeas "sex-ratio" com machos normais por sucessivas gerações, sempre obtendo excesso de fêmeas nas progênies e os raros machos sobreviventes não transmitiram o **SR** para sua prole, indicando herança exclusivamente citoplasmática. Injeções de macerados de fêmeas **SR** em fêmeas normais resultou em transferência do fenótipo, indicando como responsável um agente infeccioso. Finalmente, a estimativa da viabilidade de ovo a larva da linhagem **SR** foi metade da de uma

linhagem normal, sugerindo que os machos morrem em um estágio precoce da embriogênese e caracterizando o **SR** de *D. melanogaster* como resultante da ação de um agente androcida precoce (Montenegro *et al.*, 2000; Montenegro, 2001; ver Anexo 1).

A existência de um agente androcida em uma espécie modelo como *D. melanogaster* abre a oportunidade de se estudar o mecanismo da mortalidade de machos em um sistema com diversas técnicas e ferramentas inexistentes para outros organismos. A descoberta de um agente em populações também permite que aspectos ecológicos e evolutivos da interação entre parasita e hospedeiro sejam Entretanto, a interação entre *D. melanogaster* e seu parasita ainda não foi estudada em muito detalhe.

2) Objetivos e formato da tese

Os objetivos deste trabalho são:

1) Determinar o agente causador da mortalidade de machos em *D. melanogaster*.

2) Determinar se existem efeitos diretos do **AA** no valor adaptativo das moscas infectadas.

3) Determinar se existem efeitos ambientais na eficiência de transmissão do **AA**.

Este trabalho está apresentado em forma de artigos. Além da introdução geral, há três artigos, cada um abordando um dos objetivos acima, e uma conclusão geral. O primeiro artigo, sobre a identificação e origem do agente androcida de *D. melanogaster*, foi aceito para publicação na revista Insect Molecular Biology. O segundo artigo, sobre efeitos de *Spiroplasma* e *Wolbachia* no valor adaptativo das fêmeas hospedeiras, está em fase final de preparação e deverá ser submetido para publicação em breve. O terceiro, sobre efeitos da temperatura na eficiência de transmissão do agente androcida de *D. melanogaster*, foi publicado no periódico Journal of Invertebrate Pathology. No anexo 1 está o artigo, publicado no periódico Heredity, de meu trabalho de mestrado.

3.1) Artigo 1

Título:

Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*

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Male-killing *Spiroplasma* naturally infecting *Drosophila melanogaster*

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Abstract

Elucidation of the mechanism of action of selfish genetic elements is difficult outside species with well-defined genetics. Male-killing, the phenomenon whereby inherited bacteria kill male hosts during embryogenesis, is thus uncharacterized in mechanistic terms despite being common and important in insects. We characterized the prevalence, identity and source of the male-killing infection recently discovered in *Drosophila melanogaster* in Brazil. Male-killing was found to be present in 2.3% of flies from Recife, Brazil, and was uniquely associated with the presence of *Spiroplasma* infection. The identity of sequences across part of the 16S and across the 16S–23S ITS region indicated that the male-killing infection of *D. melanogaster* was very closely related to *S. poulsonii*, the source of the male-killing infection in *willistoni* group flies also found in South America. The sequences of two further protein-coding genes indicated the *D. melanogaster* infection to be most closely related to that found in *D. nebulosa*, from the *willistoni* group. Our data suggest that the establishment of *D. melanogaster* in South America was associated with the movement of male-killing bacteria between species.

Keywords: male-killing, *Drosophila melanogaster*, *Spiroplasma*, *Wolbachia*.

Introduction

For over 100 years, *Drosophila melanogaster* has served as a model for diverse fields in biology, such as classical genetics, ecology, evolution and developmental biology (Rubin, 1988; Powell, 1997). It has also been used to elucidate the mechanisms and importance of a range of selfish genetic elements, such as transposable elements (Ruiz & Carareto, 2003), meiotic drive genes (e.g. segregation distorter (SD): Merrill *et al.*, 1999; Houtchens & Lyttle, 2003) and cytoplasmic incompatibility caused by the bacteria *Wolbachia* (Reynolds & Hoffmann, 2002; Hoffmann *et al.*, 1998). Male-killing, a phenotype associated with maternally-transmitted bacteria that is found widely in insects, is somewhat of an exception in this regard because of the lack of a naturally occurring male-killing infection. Past work has been limited to examining artificial transinfections in *D. melanogaster*, which differ in strength of phenotype from natural infections (Anbutsu & Fukatsu, 2003; Hurst *et al.*, 2003).

Male-killing bacteria have been described in a plethora of insect hosts, spanning more than thirty species from six insect orders (see review in Hurst & Jiggins, 2000). In the *Drosophila* genus, ten species have been found infected with male-killing agents: *D. bifasciata* (Hurst *et al.*, 2000), *D. borealis* (Carson, 1956), *D. prosaltans* (Cavalcanti *et al.*, 1958), *D. robusta* (Poulson, 1966), *D. willistoni*, *D. equinoxialis*, *D. paulistorum* and *D. nebulosa* (Williamson & Poulson, 1979), *D. innubila* (Jaenike *et al.*, 2003) and *D. roehrae* (Vaz *et al.*, 1998). However, none of these host–parasite interactions have been described in much detail, mainly because these species do not have the genetic tools found in *D. melanogaster*. Indeed, the most studied male-killing system in *Drosophila* to date is *Spiroplasma poulsonii*, the agent found in *D. willistoni* and artificially introduced into *D. melanogaster* (Sakaguchi & Poulson, 1963; Williamson & Poulson, 1979). However, whilst transinfected spiroplasmas were successfully maintained in *D. melanogaster*, they never achieved the strength of phenotype and high transmission efficiency found in the natural host (Counce & Poulson, 1966).

Recently, an unidentified male-killing agent was recorded in some natural populations of *D. melanogaster* in Brazil described as a maternally heritable condition where strong female-biased sex ratios were combined with presence of a low egg hatch rate, a trait that is infectious following microinjection of haemolymph (Montenegro *et al.*, 2000). This male-killing agent, termed 'MSRO' for *m*elanogaster *s*ex *r*atio *o*rganism, presents an opportunity to study a natural strain of male-killer infecting *D. melanogaster* and we

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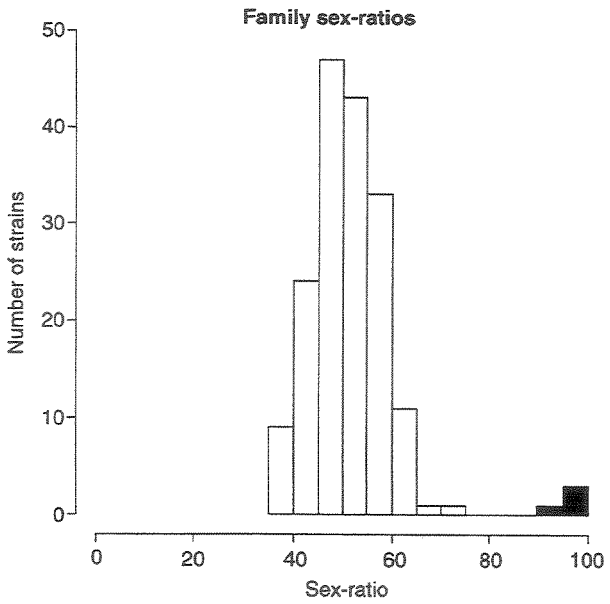

Figure 1. Sex ratio (%female) of the broods of 173 flies with broods > 10 collected in Recife, Brazil. Black bars represent male-killer infected flies, white bars represent flies with even sex ratio.

therefore investigated both its prevalence in the field and its taxonomic nature. We concluded that this bacterium is present in around 2% of wild flies in Brazil, and that the agent causing the trait is a *Spiroplasma* bacterium very closely related to that found in *D. nebulosa*, a neotropical fly with which *D. melanogaster* became sympatric following its recent colonization of the New World. This indicates that the range expansion of *D. melanogaster* is likely to have been associated with exchange of a male-killing bacterium.

Results

Male-killing frequency

One hundred and seventy-three *D. melanogaster* females that produced > ten F_1 progeny were isolated from the Recife population, of which 169 produced more than twenty F_1 flies. Of these 173, four produced a female-biased sex ratio and 169 a normal 1 : 1 sex ratio (Fig. 1). These data allowed us to estimate the prevalence within *D. melanogaster* as 2.3% (95% CI: 0.6–5.8%). The four male-killer infected lines were statistically homogenous for sex ratio (RED-42, 35 females and 0 males; RED-67, 62 females and 0 males; RED-85, 51 females and 0 males; RED-104, 57 females and 3 males; test for heterogeneity, $P = 0.062$, ns), and the sex ratio of these broods altogether was 98.56% female. Egg hatch rate was homogeneous among male-killer infected lines (Fisher's exact test, $P = 0.853$), and average hatch rate was 42% ($n = 400$). Maternal inheritance of the all-female trait was confirmed in each of these lines through five generations. In contrast, the sex ratio produced by normal flies was homogeneous (Pearson's chi-square, $\chi^2 = 185.38$, df = 168, $P > 0.05$). The sex ratio of these broods taken as one was 50.69% female (6431 ♀ and 6255 ♂, Fig. 1), which does not differ statistically from a 1 : 1 sex ratio ($\chi^2 = 2.44$, 1 df; $P > 0.05$). The egg hatch rate produced by normal flies was 89% ($n = 100$).

Male-killer identification

From the initial screen using the primers SpoulF and SpoulR that amplify members of the genus *Spiroplasma*, all four Recife male-killing lines tested positive for *Spiroplasma* presence. However, there was no signal in any of fourteen normal Recife strains tested, despite this template being amplifiable using general insect COI primers (Fisher's exact test for association between male-killing and presence of *Spiroplasma*, $P = 0.0079$). Further corroboration of this association is derived from the observation that the male-killing lines previously isolated from Campinas and two Canton-S lines transinfected by microinjection (Montenegro *et al.*, 2000) were positive for presence of *Spiroplasma*, whereas the Canton-S base stock that into which the transinfection was established was negative. Finally, one of the original male-killing strains from Recife was spontaneously cured in the laboratory. Whilst the strain maintaining the male-killer tested positive for *Spiroplasma*, the cured strain tested negative for it. From these combined data we can conclude with high certainty that male-killing and *Spiroplasma* presence are associated.

Within male-killing lines, amplification with the primers *wsp81F* and *wsp691R* revealed the presence of *Wolbachia* in all the four Recife lines infected with a male-killer (see above), but not in the Campinas male-killer infected line. The fact that *Wolbachia* was not necessary for male-killing is confirmed by three observations. First, *Wolbachia* was absent from male-killer transinfected Canton-S lines. Second, *Wolbachia* was retained in the Recife male-killer line that spontaneously cured in the laboratory, despite loss of the male-killing trait from this line. Third, Recife lines producing a normal sex ratio also commonly carried *Wolbachia*: thirty-four out of thirty-six normal lines tested were *Wolbachia* positive. Thus, *Wolbachia* occurs across normal and male-killer infected lines in Recife, is present even after curing of the male-killing trait, and is not in any way associated with the presence of male-killing. As a point to note, the *wsp* sequence of the *Wolbachia* strain in Recife *D. melanogaster* was identical to that found previously in male and female *D. melanogaster* (Zhou *et al.*, 1998).

Notwithstanding the perfect association between *Spiroplasma* presence and the presence of the male-killing trait, which holds across transinfected as well as naturally infected lines, it is possible that there are other infections associated with male-killing. We tested whether other infections were present by examining the proportion of product obtained with 16S rDNA general primers that are derived

from *Spiroplasma* from flies of the Campinas and transinfected lines. The percentage of clones obtained from PCR product using general eubacterial primers that possessed a *Spiroplasma* insert was 95.7% for the Campinas male-killer infected line (45 spiroplasma-positive out of 47 successful reactions, 1 reaction failed) and 92.3% in the transinfected line (48 spiroplasma-positive out of 52 successful reactions, 5 reactions failed). Overall, the percentage of 16S rDNA clones with a spiroplasma insert is 93.9%. This very high figure indicates there are no other common infections within these male killing lines, and that the causal agent of male-killing is a *Spiroplasma*.

Phylogenetic affiliation of the melanogaster Spiroplasma *and* Wolbachia

We amplified and sequenced a section of the 16S rDNA and the whole 16S–23S ITS sequence of the MSRO *Spiroplasma* from *D. melanogaster* (EMBL accession nos. AJ631998 and AJ631999). These were identical over all 1089 bases to that previously ascertained for the male-killing infection NSRO (nebulosa sex ratio organism) isolated from *D. nebulosa* and for the *S. poulsonii* type strain (a male-killer isolated from *D. willistoni*, Williamson *et al.*, 1999). According to the 2.5% rule of species delineation used in bacterial taxonomy (Stackebrandt & Goebel, 1994), this indicates that the spiroplasma from *D. melanogaster* should be considered *S. poulsonii sensu lato*.

In order to resolve the affiliation of the MSRO strain more closely with respect to ancestry within the *S. poulsonii* clade, we obtained the sequence of the *spoT* and *p58* genes from the MSRO strain (from *D. melanogaster*), the NSRO strain (from *D. nebulosa*), the *S. poulsonii* type strain (from *D. willistoni*), and two closely related non-male-killing species of spiroplasma (*S. insolitum* and *S. phoeniceum*) that live in other insect species and compared these with the known sequences of these genes from *S. citri* and *S. kunkelii*.

The sequences of both the *spoT* and *p58* genes from MSRO (the melanogaster strain) and NSRO (the nebulosa strain) were identical across 1306 bases of sequence, but these differed from the *S. poulsonii* type strain isolated from *D. willistoni* (Tables 1 and 2 for *spoT* and *p58*, respectively).The three *Drosophila Spiroplasma* strains clearly formed a monophyletic group with respect to other *Spiroplasma* species for both the *spoT* and *p58* genes, reinforcing the identity of MSRO and NSRO as *S. poulsonii s.l.* (Figs 2 and 3). These trees also indicate that the melanogaster spiroplasma strain MSRO is most closely related to strain NSRO (from *D. nebulosa*) rather than the *S. poulsonii* type strain from *D. willistoni*. Second, the

Table 1. Number of differences in nucleotides between *Spiroplasma* species (above diagonal) and Kimura 2-parameter distances with pair-wise deletion of gaps and missing data calculated using MEGA2.1 (below the diagonal), for 513 bp of the *spoT* gene

	NSRO-Guadalupe*	MSRO-Campinas†	MSRO-Recife‡	*S. poulsonii*	*S. insolitum*	*S. phoeniceum*	*S. citri*	*S. kunkelii*
NSRO-Guadalupe	x	0	0	3	36	51	54	54
MSRO-Campinas	0.0000	x	0	3	36	51	54	54
MSRO-Recife	0.0000	0.0000	x	3	36	51	54	54
S. poulsonii	0.0059	0.0059	0.0059	x	37	52	55	55
S. insolitum	0.0742	0.0742	0.0742	0.0765	x	57	58	58
S. phoeniceum	0.1075	0.1075	0.1075	0.1099	0.1209	x	27	5
S. citri	0.1141	0.1141	0.1141	0.1165	0.1231	0.0550	x	28
S. kunkelii	0.1145	0.1145	0.1145	0.1170	0.1233	0.0098	0.0572	x

*NSRO-Guadaloupe – *Spiroplasma* from *D. nebulosa* collected in Guadaloupe.
†MSRO-Campinas – *Spiroplasma* from *D. melanogaster* collected in Campinas, Brazil.
‡MSRO-Recife – *Spiroplasma* from *D. melanogaster* collected in Recife, Brazil.

Table 2. Number of differences in nucleotides between *Spiroplasma* species (above diagonal) and Kimura 2-parameter distances with pair-wise deletion of gaps and missing data calculated using MEGA2.1 (below the diagonal), for 793 bp of the *p58* gene

	NSRO-Guadalupe*	MSRO-Campinas†	MSRO-Recife‡	*S. poulsonii*	*S. insolitum*	*S. phoeniceum*	*S. kunkelii*
NSRO-Guadalupe	x	0	0	7	28	35	28
MSRO-Campinas	0.0000	x	0	7	28	35	28
MSRO-Recife	0.0000	0.0000	x	7	28	35	28
S. poulsonii	0.0089	0.0089	0.0089	x	29	38	31
S. insolitum	0.0362	0.0362	0.0362	0.0375	x	28	32
S. phoeniceum	0.0456	0.0456	0.0456	0.0496	0.0363	x	39
S. kunkelii	0.0360	0.0360	0.0360	0.0402	0.0416	0.0511	x

*NSRO-Guadaloupe – *Spiroplasma* from *D. nebulosa* collected in Guadaloupe.
†MSRO-Campinas – *Spiroplasma* from *D. melanogaster* collected in Campinas, Brazil.
‡MSRO-Recife – *Spiroplasma* from *D. melanogaster* collected in Recife, Brazil.

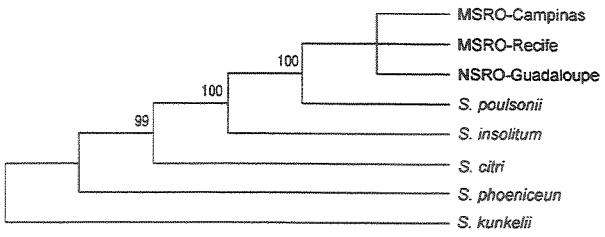

Figure 2. Consensus maximum parsimony tree of the *spoT* gene from *Spiroplasma poulsonii* and relatives created with MEGA2.1, using the max–min branch and bound method, with 1000 bootstrap replicates. Data missing in one or more taxa were deleted in all taxa. Numbers at branches indicate percentage of bootstrap support for particular nodes.

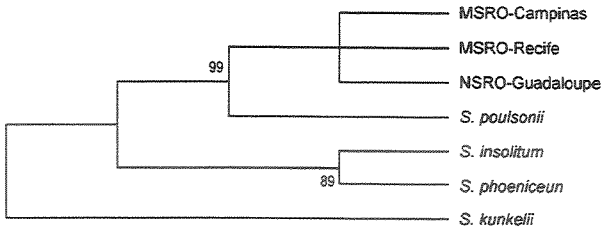

Figure 3. Consensus maximum parsimony tree of the *p58* gene from *Spiroplasma poulsonii* and relatives created with MEGA2.1, using the max–min branch and bound method, with 1000 bootstrap replicates. Data missing in one or more taxa were deleted in all taxa. Numbers at branches indicate percentage of bootstrap support for particular nodes.

lack of any differentiation in DNA sequence indicates a very recent common ancestry of the MSRO and NSRO strains (accession numbers. For *spoT D. melanogaster* spiroplasma MSRO: AJ628443, *D. nebulosa* spiroplasma NSRO: AJ628442, *S. poulsonii* ATCC^T from *D. willistoni*: AJ628441, *S. insolitum*: AJ628440, *S. phoeniceum*: AJ628439; for *p58*, *D. melanogaster* MSRO strain: AJ628434, *D. nebulosa* NSRO strain: AJ628435, *S. poulsonii* ATCC 43153^T from *D. willistoni*: AJ628436, *S. insolitum*: AJ628437, *S. phoeniceum*: AJ628438).

We additionally sequenced the *fru* gene for the MSRO and NSRO strains and for *S. insolitum*. Whilst the *fru* gene was almost twenty-two times as divergent as 16S rDNA between MSRO and that of *S. insolitum* (four differences in 673 bp for the 16S rDNA compared to forty-seven differences and one gap in 360 bp for *fru*), it was identical between MSRO and NSRO strains, further indicating recent common ancestry (accession numbers: *S. insolitum*: AJ631997, *D. melanogaster* spiroplasma: AJ628444, *D. nebulosa* spiroplasma: AJ628445).

Discussion

Drosophila melanogaster is a useful tool in elucidating the mechanistic basis of interactions, and in examining questions in evolutionary ecology. Indeed, *D. melanogaster* has proven pivotal in studies of selfish genetic element mechanisms and population biology, with elucidation of the mechanism of segregation distortion (Merrill *et al.*, 1999; Houtchens & Lyttle, 2003) and transposable element action (Timakov *et al.*, 2002), as well as insights into the basis of variation in the intensity of *Wolbachia*-induced cytoplasmic incompatibility (Reynolds & Hoffmann, 2002; Hoffmann *et al.*, 1998). One area which previously lacked study is interaction with male-killing inherited bacteria. In this study, we record the presence of a male-killing *Spiroplasma* infection in 2.3% of flies collected from Recife, Brazil. Molecular systematic data indicated this *Spiroplasma* should be considered *S. poulsonii s.l.*, and is affiliated with the male-killer infection found in New World *willistoni* group flies.

In a classical example of horizontal transmission, it has been demonstrated that *P*-elements were transmitted from *D. willistoni* to *D. melanogaster*, with the mite *Proctolaelaps regalis* suggested as a possible vector (Daniels *et al.*, 1990; Houck *et al.*, 1991). This transmission is known to have occurred recently, because *D. melanogaster* (an Old World species) and *D. willistoni* (a New World species) were allopatric until the anthropogenic introduction of *D. melanogaster* to the New World about 300 years ago (Lachaise *et al.*, 1988; Powell, 1997; Lachaise & Silvain, 2004). Given that this clade of *Spiroplasma* is not known outside *Drosophila*, our case study provides a second case where the introduction of *D. melanogaster* into the New World has been associated with movement of an inherited parasite, in this case a *Spiroplasma* male-killer bacterium. In this case, phylogenetic evidence from two *Spiroplasma* genes indicates the movement between *D. nebulosa* and *D. melanogaster*, and the identity of the two strains over three protein-coding gene sequences (1771 bp) corroborates the recent common ancestry of the two strains. It should be noted that *D. nebulosa* is a close ally of *D. willistoni*, and falls within the *willistoni* group of flies. It too has cohabited with *D. melanogaster*, both geographically and ecologically, since the spread of *D. melanogaster* into the New World (Vilela *et al.*, 1980; Tidon-Sklorz *et al.*, 1994; Medeiros & Klaczko, 2004).

Given that spiroplasma infection is known to be ancient in the *willistoni* group including *D. nebulosa* and that this clade of *Spiroplasma* is only currently known in *Drosophila*, the most likely direction of movement is from *D. nebulosa* to *D. melanogaster*. Because the *willistoni* and *melanogaster* species groups were allopatric until recently, the alternative direction would require movement of the *Spiroplasma* from a member of the *D. willistoni* group into an intermediate host within the New World at some point in the past, movement of the intermediate host from the New World to the Old World, infection of *D. melanogaster* from the intermediate host in the Old World, followed by transfer back into *D. nebulosa* in the New World associated with the introduction of *D. melanogaster* to the New World. Because this

represents a less parsimonious explanation for the data, we favour the hypothesis that movement occurs from *D. nebulosa* to *D. melanogaster*. Thus we postulate that the expansion of the range of *D. melanogaster* has made it subject to invasion by the *Spiroplasma* from *D. nebulosa* in addition to the *P*-element from *D. willistoni*. Changes in species range, both anthropogenic and natural, are associated not just with exposure to new classical parasites, but also to a diverse array of new 'inherited' and genomic parasites. The presence of body mites on *D. nebulosa* in wild populations (G. Hurst, pers. obs.) also makes mites a tempting prospect as a vector for the bacterium between species.

The movement of *P*-elements into *D. melanogaster* in the New World was followed by the spread of these elements through the New World and then across the Old World (Anxolabéhère *et al.*, 1988). In contrast, male-killing has never been reported elsewhere (e.g. Australia, A. Hoffmann, pers. comm.). As male-killing in *D. melanogaster* is a temperature-sensitive trait, being cured by low temperatures (Montenegro & Klaczko, 2004), it is unlikely that it will be found outside the tropics, and even there its prevalence and range could decline and contract in the winter. We thus predict that the effect of this invasion will be dissimilar to that seen for *P*-elements in this regard.

Previous data have indicated that the infection in *D. melanogaster* is more stable than the existing NSRO strain spiroplasma transinfected into *D. melanogaster* that is currently used in experiments on the mechanism and dynamics of male-killing. Transmission efficiency is higher, and the infection is stable down to 20 °C rather than 23 °C in *D. willistoni* and *D. nebulosa* (Montenegro *et al.*, 2000; Montenegro & Klaczko, 2004). It therefore gives the opportunity to study the full phenotype of a male-killer in the model organism *D. melanogaster*. It will also be useful as a model for investigating the dynamics of male-killers in *Drosophila*, which have so far proved somewhat enigmatic (Ebbert, 1991; Hurst & Majerus, 1993). Prevalence of the infection at 2.3% parallels that found in the *willistoni* group, where infection prevalence varies between 0.5 and 6.0%. In future, we can use *D. melanogaster* to try and identify whether and what advantages of male-killing to the bacterium exist in this species.

Experimental procedures

Source of flies and spiroplasma genomic DNA

Flies were collected from the wild during January 2003 at Recife, Pernambuco State, Brazil. The collection was performed at one single site over three days. Females from the melanogaster group were placed individually in small vials with approximately 15 ml medium. After five days they were moved to new vials, and five days later they were finally discarded. *D. melanogaster* and *D. simulans* were distinguished by the external morphology of the male genital arch. *D. simulans* were discarded.

In addition to flies from Recife, the male-killer-infected line collected during 1997 in Campinas, São Paulo State (2665 km from Recife), and two artificially transinfected male-killer-infected lines obtained by injecting macerates of this strain into Canton-S females (Montenegro *et al.*, 2000), were also used in molecular analysis. Within the analyses performed in this study, we also utilized template from *D. nebulosa* from Guadaloupe infected with *Spiroplasma* (Bentley *et al.*, 2002). Genomic DNA of pure *S. poulsonii* ATCC 43153^T, [the male-killer in *D. willistoni* (Williamson *et al.*, 1999)], *S. insolitum* and *S. phoeniceum* were also analysed (DNA templates kindly provided by Gail Gasparich, Towson University).

Male-killer identification

DNA from each fly line was extracted from two flies macerated in 100 µl 5% Chelex 100 resin. To this was added 20 µg proteinase K, incubated at 37 °C overnight. Samples were then heated to 95 °C for 10 min to denature the proteinase K and centrifuged for 1 min at 10 000 *g*. This DNA was used for PCR amplifications with *Spiroplasma* and *Wolbachia* specific primers. Primers used were SpoulF (5′-GCT TAA CTC CAG TTC GCC-3′) and SpoulR (5′-CCT GTC TCA ATG TTA ACC TC-3′) for *Spiroplasma*, and wsp81F and wsp691R, general for all *Wolbachia* (Zhou *et al.*, 1998). Amplification of the insect mitochondrial *cytochrome-oxidase I* gene was also performed for all strains following Brunton & Hurst (1998), to establish whether negative results were the result of poor DNA quality.

In order to quantify the representation of candidate bacteria in hosts infected with the male-killing trait, 'clean' DNA from ovaries of two individuals was obtained, followed by amplification and cloning of a fragment of eubacterial 16S rDNA using a primer pair that amplifies this region from a wide range of eubacteria. Clones were subsequently checked for insert identity to establish the representation of *Spiroplasma* in the bacterial flora of the fly. In brief, ovaries were dissected in *Drosophila* Ringer solution and DNA extracted with the DNeasy™ Tissue Kit (Qiagen, Valencia, CA, USA). The general eubacterial primers 792f and 1495r were then used to amplify a fragment of approximately 700 bp from these templates. The product was then purified using the Wizard® SV Gel PCR Clean-up System (Promega, Madison, WI, USA) and the purified product ligated into pGEM-T Easy Vector (Promega), and transformed into *Escherichia coli* DH5α. Successfully transformed (white) colonies were replica plated and left to grow overnight at 37 °C. The proportion of these clones bearing *Spiroplasma* inserts was estimated with a PCR reaction using primers M13f and M13r, which amplify the flanking regions of the vector, and internal primer SpoulR. Plasmids with a 16S rDNA *Spiroplasma* insert should amplify fragments of approximately 310 or 430 bp in addition to a larger fragment of 960 bp, which will be the only band present if *Spiroplasma* 16S rDNA is not present. As a control, a fragment of *Wolbachia* 16S rDNA, inserted in the same plasmid, was used.

Phylogenetic affiliation of the melanogaster Spiroplasma *and* Wolbachia

In order to establish the general position of the *D. melanogaster Spiroplasma* in relation to other *Spiroplasma* infections, part of the 16S rDNA of one clone from the Campinas strain and one clone from the transinfected strain were sequenced. In addition, the 16S–23S ITS region of *Spiroplasma* template from the Campinas male-killer infected strain and from one of the Recife male-killer

infected strains was amplified using primers JO4 and N2 (Schulenburg *et al.*, 2000), and then directly sequenced.

In order to obtain sequences of more variable protein-coding genes, genes within a small shotgun sequence library of *S. poulsonii* type strain were examined, and compared with homologues in the *S. kunkelii* and *S. citri* draft genomes. Two genes, one with similarity to the (p)ppGpp 39-pyrophosphohydrolase *spoT* gene (Jacob *et al.*, 1997) and the other with similarity to the putative adhesin *p58* gene (Ye *et al.*, 1997), showed substantial sequence divergence between the three genomes compared to the 16S/ITS/23S block. Primers for PCR amplification were then designed across conserved regions of these genes. For *spoT*, primer pair spoT-f (5′-CAA ACA AAA GGA CAA ATT GAA G-3′) and spoT-r (5′-CAC TGA AGC GTT TAA ATG AC-3′) was used with the PCR profile consisting of an initial cycle of 2 min at 94 °C, followed by thirty cycles of 20 s at 94 °C, 60 s at 54 °C and 55 s at 72 °C. For *p58* primer pair p58-f (5′-GTT GGT TGA ATA ATA TCT GTT g-3′) and p58-r (5′-GAT GGT GCT AAA TTA TAT TGA C-3′) was used with the same PCR profile as for the *spoT* gene. PCR products were then directly sequenced. A fragment spanning two other genes, with similarity to the fructose-specific IIABC component *fruA* and the transcriptional regulator *fruR* of *S. citri* (Gaurivaud *et al.*, 2000) was also obtained for MSRO, for NSRO and for *S. insolitum*. Primer pair fru-f (5′-GTC ATA ATT GCA ATT GCT GG-3′) and fru-r (5′-CAA TGA TTA AAG CGG AGG T-3′) was used with the PCR profile consisting of an initial cycle of 2 min at 92 °C followed by thirty-six cycles of 20 s at 92 °C, 60 s at 54 °C and 30 s at 72 °C.

The phylogenetic affiliation of the *Wolbachia* strain infecting Recife lines was ascertained from the *wsp* sequence. DNA template from three Recife flies, two uninfected with male-killers, and one infected, was amplified using primer pair wsp81f/691r and directly sequenced.

Phylogenetic analysis

The sequences given above were examined by eye, and the two strands compared to create a consensus sequence, which was then checked for premature termination codons. The sequences from different hosts were then aligned using ClustalW (Thompson *et al.*, 1994) and this alignment checked by eye. The aligned sequences were 800 bp long for *p58* (including seven sites with missing data) and 513 bp for *spoT*. There were no gaps in the alignment of either gene. Distances between each of the sequences were estimated with the Kimura 2-parameter model with the 'complete deletion' option implemented in Mega version 2.1 (Kumar *et al.*, 2001). Phylogenetic trees were constructed by the maximum parsimony method with Mega, using max-mini branch-and-bound method and complete deletion. Indices of support for particular nodes on the tree were obtained running 1000 heuristic bootstrap replicates. A 50% majority rule tree was constructed on the basis of bootstrap support. The number of parsimony-informative sites was twenty-two for the *p58* and fifty-four for the *spoT* sequences.

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3.2) Artigo 2

Título:

Fitness effects of *Wolbachia* and *Spiroplasma* in *Drosophila melanogaster*

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Fitness effects of *Wolbachia* and *Spiroplasma* in *Drosophila melanogaster*

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Abstract

Maternally inherited endosymbionts that manipulate the reproduction of their insect host are very common. Aside from the reproductive manipulation they produce, the fitness of these symbionts depends in part on the direct impact they have on the female host. Although this parameter has commonly been investigated for single infections, it has much more rarely been established in dual infections. We here establish the direct effect of infection with two different symbionts exhibiting different reproductive manipulation phenotypes, both alone and in combination, in the fruit fly *Drosophila melanogaster*. This species carries a cytoplasmic-incompatibility inducing *Wolbachia* and a male-killing *Spiroplasma*, occurring as single or double (co-) infections in natural populations. We assessed fitness effects of these bacteria on the host, by comparing larval competitiveness and adult fecundity of Uninfected, *Wolbachia*, *Spiroplasma* and *Wolbachia-Spiroplasma* coinfected females. We found no effect of infection status on the fitness of females for both estimates, that is, no evidence of any benefits or costs to either single or co-infection. This leads to the conclusion that both bacteria probably have other sources of benefits to persist in *D. melanogaster* populations, either by means of their reproductive manipulations (fitness compensation from male death in *Spiroplasma* and cytoplasmic incompatibility in *Wolbachia*) or by positive fitness interactions on other fitness components.

Word count: 3424

Keywords: *Wolbachia*, *Spiroplasma*, male-killing, symbiosis, virulence, fecundity.

Running title: *Wolbachia* and *Spiroplasma* fitness effects.

Introduction

Microorganismal endosymbionts of arthropods are extremely common, living in close and often obligatory relationship with their hosts. A subset of these microoganisms, inherited symbionts, pass from an infected female to her progeny through the cytoplasm of eggs. The maternal vertical transmission of these microorganisms, which include bacteria and protists, places them in an interesting and contradictory position: in the first place, vertical transmission produces a covariance between microbe and female host fitness, and selection towards being less virulent, and if possible, beneficial to female hosts (Fine, 1975; Ewald, 1987; Yamamura, 1993; Lipsitch et al., 1995). In the second place, the exclusive maternal transmission of some of these symbionts creates a reproductive asymmetry that can be exploited: males are 'dead ends' for the symbionts, consequently the microorganisms experience selection to alter host reproduction to increase the net production of females, the transmitting sex (Eberhard, 1980; Cosmides & Tooby, 1981). Several alterations of arthropod reproduction have been described, such as cytoplasmic incompatibility, feminisation, parthenogenesis induction and male-killing (reviews in O'Neill, Hoffmann & Werren, 1997; Werren, 1997; Hurst & Jiggins, 2000; Bandi et al., 2001).

Reproductive parasites face a dilemma: their fitness is strongly correlated with female host fitness, so there is selection not to harm the female host (Fine, 1975; Ewald, 1987; Yamamura, 1993; Lipsitch et al., 1995). However, there can be a trade-off between bacterial titre and both strength of reproductive manipulation (e.g. proportion of infected males that are killed) and vertical transmission efficiency. For instance, bacterial load is positively correlated with high transmission and strength of male-killing in *Drosophila melanogaster* (Anbutsu & Fukatsu, 2003) and with strength of cytoplasmic incompatibility in (Veneti et al., 2003, 2004). Given that there

is a correlation between bacterial titre and cost to the host (Min & Benzer, 1997; McGraw et al., 2002; Mouton et al., 2004), there can therefore be a trade-off for the microorganisms between cost of infection and strength of phenotype / efficiency of vertical transmission.

This trade-off means the microorganism's optimal phenotype does not necessarily match that of the female host. Mild virulence (associated with better transmission or more efficient reproductive manipulation) may be a selective optimum for the microorganism. This produces a spectrum of possible interactions between inherited parasites and their female hosts, from being directly deleterious (through high titre being required to establish a strong phenotype or high transmission efficiency) through neutral (where a strong phenotype and high transmission efficiency occurs at low titre, or where the host has driven down bacterial titre) to beneficial (where the host has either become dependent on microbe presence, or the microbe plays a role in host physiology). All possible direct effects on fitness have been found: deleterious in *D. bifasciata* (Ikeda, 1970) and *D. willistoni* (Ebbert, 1991, 1995); no effect: *Wolbachia* in *Acraea encedon* (Jiggins et al., 2002) and CI *Wolbachia* in *D. melanogaster* (Fry, Palmer & Rand, 2004); and beneficial: CI *Wolbachia* in *D. melanogaster* (Fry & Rand, 2002; Fry, Palmer & Rand, 2004) and *Aedes albopictus* (Dobson, Rattanadechakul & Marsland, 2004). This diversity of outcomes is not a surprise, given the conflicting forces accounting for the evolution of the interactions between hosts and parasites.

The above analyses were based on the premise that a single strain of a bacterium infected a host. Models of multiple infections show that two different vertically transmitted parasites can only persist in a panmitic population if there are double-infected hosts, either for two different *Wolbachia* strains inducing cytoplasmic

75 incompatibility (Frank, 1998) or one male-killing strain and one cytoplasmic
76 incompatibility inducing strain (Engelstädter, Telschow, & Hammerstein, 2004). It is
77 common to observe more than one strain infecting a single host organism. For
78 instance, there can be up to five strains of *Wolbachia* within a host individual in the
79 ant *Formica exsecta* (Reuter & Keller, 2003). Although co-infections of bacteria that
80 produce a common phenotype have been long recognised, co-infection of cytoplasmic
81 incompatibility *Wolbachia* with feminizers (Hiroki et al., 2004), and male-killers
82 (Montenegro et al., in press) have recently been recorded.

83 The presence of co-infection introduces the possibility that interactions
84 between different bacterial strains will affect the titre of each other (e.g. Mouton et al.,
85 2004; Kondo et al., submitted), and therefore virulence. There is also the possibility of
86 non-additive effects of existing titres on the cost suffered by the host. Thus, the
87 expectation of cost from a single infection may be different than for co-infections. In
88 this paper, we examine the cost of infection for the previously uninvestigated class of
89 co-infection where there the bacteria cause distinct phenotypes on their hosts. Our
90 study system is *Drosophila melanogaster,* a known host of a *Wolbachia* strain that
91 causes mild cytoplasmic incompatibility (Solignac et al., 1994; Hoffmann, Hercus &
92 Dagher, 1998). More recently, it has been observed to be host of a male-killing
93 *Spiroplasma* (Montenegro et al., 2000; in press). These two bacteria also have been
94 found to co-infect the same female in some populations (Montenegro et al., in press).
95 Here we investigated the larval competitiveness and adult fecundity of female flies
96 infected with either *Spiroplasma* or with *Wolbachia*, as well as *Spiroplasma-*
97 *Wolbachia* co-infected females, and compared them to uninfected flies of identical
98 genotype. We assess these for hosts reared at low stress (low larval density) and high
99 stress (high larval density) to detect any condition dependent virulence. In addition to

100 bringing insight into the factors affecting virulence, this study will also allow us to
101 understand the factors stabilizing this type of dual infection.
102
103 ## Materials and Methods
104 ***Drosophila melanogaster* strains:** the original stocks were an uninfected
105 Canton-S stock (henceforth U cytotype); the *Spiroplasma*-infected strain (S cytotype)
106 collected in 1997 in Campinas, Brazil (Montenegro et al., 2000), one *Wolbachia*-
107 infected isofemale strain (W cytotype) collected in Recife, Brazil; and another
108 isofemale strain from the same Recife collection, double infected with *Spiroplasma*
109 and *Wolbachia* (W+S cytotype) (Montenegro et al., in press). Both S and W+S male-
110 killing strains have been maintained by crossing with Canton-S males, S for more
111 than 40 generations and W+S, more than 8 generations, rendering the S and W+S
112 genetic backgrounds effectively Canton-S. Prior to beginning the larval competition
113 experiment, W females were crossed with U males for seven generations, making the
114 nuclear genotype effectively Canton-S but maintaining the original cytotype and,
115 consequently, the *Wolbachia* infection status.
116 **Larval competition:** a comparative measure of larval viability was assessed
117 by competing known numbers of individuals of the different cytotype lines against the
118 *sparkling poliert* (*spa*) eye colour mutant strain. First instar larvae were collected
119 from mass crosses of each one of the cytotypes and from *spa* mass crosses. The
120 experiment was performed at two larval densities, nine (three wild type plus six *spa*)
121 and 36 (12 wild type plus 24 *spa*) larvae per vial containing 1ml of food. Food was
122 standard SY medium (100g yeast, 20g agar, 100g sugar, 1000ml water, 30ml nipagin
123 10% and 3ml propanoic acid) without addition of live yeast to ensure uniformity of
124 food amount between vials. Twenty-eight replicate vials of each treatment (cytotype

and larval density) were set up, and the number and eye phenotype (either wild type or *sparkling poliert*) of emerging adults from each vial was recorded. Twenty W and W+S females (ten each cytotype) were later assayed for *Wolbachia* with a PCR reaction using *wsp81 / wsp691* pair of primers to ensure the infection had been maintained. Because of the non-normality of the data, a non-parametric two-way analysis of variance was conducted on the rank of the relative ratio of wild type/(*sparkling poliert* + wild type) emerging adults, with cytotype and larval density as fixed main effects.

Adult female fecundity: prior to beginning this experiment, W and W+S strains were assayed for *Wolbachia* with the same procedure as above. To obtain a uniform genetic background and eliminate maternal effects, U, S and W+S females were crossed with W males, and W females were crossed with U males, resulting in all strains having an identical hybrid nuclear background. Subsequently, 4 days old virgin females of each cytotype were crossed with U males and allowed to oviposit on grape juice laying plates. First instar larvae from each one of these crosses were collected and transferred to vials with 1ml of SY medium at low (seven larvae per vial) and high (21 larvae per vial) densities, without addition of live yeast. Virgin females were collected from these vials, aged for four days, and then crossed individually to a U male in a vial with 7ml of SY food, with addition of some granules of live yeast to ensure plenty of food to the adults as well larvae, with 70 replicates for each cytotype at each density. To ensure that there would be no effect on female survival of brood size differences due to male-killing, pairs were moved to a new vial every day over 14 days, thus avoiding any competition due to larval crowding within a vial. 14 days was chosen as it encompasses the vast majority of the reproductive period of *D. melanogaster*, and is also probably the limit of survivorship in the field.

Dead male partners were replaced to ensure maximal fertility of the female. The total number of F_1 males and females emerging was scored after all flies emerged.

Generalised linear models are an extension of linear models that relax the assumptions of normality of the data, and allow modelling of exponential error distributions (McCullagh & Nelder, 1989). Because the fecundity data is strongly non-normal, the number of female progeny was analysed with a generalised linear mixed model (GLMM) using the IRREML procedure from the Biometris GenStat Procedure Library (Goedhart & Thissen, 2003) for Genstat 7.2 (Genstat 7 Committee, 2003). GLMMs are a more sophisticated class of generalized linear models (McCullagh & Nelder, 1989) that allow random effects to be fitted, thus accounting for variation arising from repeated measures of the fecundity of the same female over the 14 days. The appropriate distribution for count data is the Poisson error distribution, and, although the canonical link function is logarithm, we used a square-root link function, as it provided a better fit for the data, after inspection of fitted versus observed values and plots of the residuals. Cytotype, larval density at growth and day were considered as fixed effects, and individual female identity was modelled as random factor. Only broods of females that survived all fourteen days and with at least one daughter were included in the analysis (flies grown at low larval density: n(U) = 58, n(W) = 55, n(S) = 57, n(W+S) = 57; flies grown at high larval density: n(U) = 45, n(W) = 40, n(S) = 47, n(W+S) = 41). To test the significance of the fixed effects and their interactions, the Wald statistic was calculated when the term was included last in the model (Genstat 7 Committee, 2003). This statistic follows a χ^2 distribution.

The number of infertile females was compared with a Pearson's χ^2 test. U and W females were considered fertile only if they produced at least one daughter,

175 females that produced male but not female progeny were considered as sterile for this
176 analysis. The reasons for this is that are: 1) S and S+W flies putatively producing just
177 a few males would be scored as sterile, as these males would die and no progeny at all
178 would be scored; and 2) for the dynamics of cytoplasmic transmitted bacteria, males
179 are dead-ends, and uninfected females producing only males would have no effect on
180 the dynamics of these bacteria.

181 **Adult female survival:** Additionally, female survival over the 14 days of the
182 fecundity experiment was analysed by fitting a proportional hazards Cox model on the
183 survival time for each female, and a sequential analysis of deviance for the fit was
184 performed. The model was fitted allowing for censored data, censored survival data
185 (escaping females or death provoked by human handling). This analysis was
186 performed using R statistical software version 2.0.1 (Ihaka & Gentleman, 1996; R
187 Development Core Team, 2004).

188

189 ## Results

190 **Larval to adult viability:** two-way ANOVA performed on the rank
191 transformed relative ratio of wild type / (*sparkling poliert* + wild type) adults
192 emerging from the competition vials showed no effects of cytotype, larval density at
193 growth or cytotype versus larval density interaction on the larval to adult viability
194 (Table 1 and Figure 1).

195 **Adult female fecundity:** Wald's tests of the GLMM fixed effects showed no
196 effect of cytotype or larval density at growth, nor any interaction involving cytotype
197 on fecundity measured in terms of production of daughters, although there is an effect
198 of day and a day versus density interaction on female fecundity (Table 2 and Figure

199 2). Infertility (failure to produce any viable progeny) was homogeneous across
200 cytotypes and densities ($\chi^2 = 6.3085$, df = 7, p = 0.504).

201 The analysis of deviance performed on the Cox model revealed that cytotype
202 has no effect on female survival, but there is a strong effect of larval density on
203 survival (Table 3 and Figure 3). Adult flies which developed at the high density have
204 higher mortality over 14 days. Again, there is no cytotype versus density interaction.

205 All W and W+S flies tested prior to the larval to adult viability and adult
206 female fecundity experiments were positive for *Wolbachia* presence.

207

208 ## Discussion

209 To persist in a population, reproductive parasites manifest a wide variety of
210 manipulations of host reproduction, including increasing the production and / or
211 survival of female hosts at a cost to the survival or fertility of males. Beside the
212 advantages gained from reproductive manipulation, two other infection parameters,
213 direct effects on host fitness and vertical transmission efficiency, determine the ability
214 of the parasites to persist in natural populations, and the prevalence obtained. When
215 co-infections occur, these parameters need to be calculated for both single infections
216 and co-infections. Here we investigated direct fitness effects of two reproductive
217 parasites, a male-killing *Spiroplasma* and a strain of *Wolbachia* that is presumed to
218 induce cytoplasmic incompatibility on *Drosophila melanogaster*. Host nuclear genetic
219 background in our experiments was uniform across infection types, and we found no
220 evidence of fitness effects of these bacteria, either alone or in co-infection, when
221 measuring adult female fecundity and larval to adult viability. We found no evidence
222 that host condition affects fitness, as this absence of any effect occurred both with low
223 and high stress during host larval life.

224 Evidence for direct fitness effects caused by vertically transmitted bacteria is
225 somewhat ambiguous. In theory, as bacterial fitness is highly correlated with fitness
226 of host females, the bacteria should be selected to be harmless – or even beneficial –
227 to females. However, a trade-off between transmission efficiency of the bacteria and
228 fitness effects could exist, if high titre correlates with high transmission rate. These
229 contradictory forces generate a multitude of possible outcomes that is confirmed by
230 recent papers: all potential fitness effects have been described, from beneficial effects
231 (Dobson, Rattanadechakul & Marsland; 2004, Fry, Palmer & Rand, 2004) to no
232 effects (Bordenstein & Werren, 2000) to deleterious effects (Ebbert, 1991, 1995).
233 Although we found no effect of *Spiroplasma* and *Wolbachia* infections in our
234 study, either singly or in co-infection, other studies did observe positive fitness effects
235 of *Wolbachia* on *D. melanogaster* (Fry & Rand, 2002; Fry, Palmer & Rand, 2004),
236 and negative effects of *Spiroplasma* on *D. willistoni* hosts (Ebbert, 1991, 1995).
237 Hoffmann, Hercus & Dagher (1998), by indirect means, proposed that in *D.*
238 *melanogaster*, *Wolbachia* only persists in Australian populations due to a direct
239 fitness benefit. Our results are difficult to compare with these studies, as the observed
240 fitness effects vary widely between studies, according to the trait studied, host genetic
241 background, and environmental conditions. The most comprehensive fitness
242 comparisons in *Wolbachia* / *D. melanogaster* were concerned with survival
243 differences between infected and non-infected flies (Fry & Rand, 2002; Fry, Palmer &
244 Rand, 2004), where a positive effect of infection was generally observed. However,
245 both no (Hoffmann Clancy & Merton, 1994) and positive (Fry, Palmer & Rand, 2004)
246 effects on fecundity were also found. To add further complexity, a particular fitness
247 measure can span the whole spectrum of interactions depending on the host nuclear
248 genotype (Fry, Palmer & Rand, 2004). With respect to the effect of male-killing

249 *Spiroplasma*, we did not find the increased early fecundity described previously for
250 interaction between *S. poulsonii* and *D. willistoni* and *D. pseudoobscura* (Ebbert,
251 1991, 1995).

252 Host genetic background can be very important for determining the outcome
253 of host/parasite fitness interactions (Bordenstein & Werren, 2000; Olsen, Reynolds, &
254 Hoffmann, 2001; Fry, Palmer & Rand, 2004), and some reported fitness effects later
255 disappeared after controlling for the host genetic background (Bordenstein & Werren,
256 2000). Our experiments controlled for differences in the nuclear genotype within each
257 experiment (adult fecundity and larval competition), so that we may be confident that
258 the lack of fitness effects in these genetic lines are real. However, we can not state
259 that a lack of effect of infection on female host viability or fecundity occurs generally
260 across the genotypes found in a natural population. This would require us to either
261 investigate an array of lines in the manner conducted here, or to examine fitness
262 effects in a genetically heterogeneous population.

263 What is the expected evolutionary pathway from multiple infections in one
264 host species? If there are no co-infected individuals, the parasite with higher relative
265 fitness will drive the others out of the population (Engelstädter, Telschow, &
266 Hammerstein, 2004; Frank, 1998). However, in populations where co-infection
267 occurs, co-infected, singly infected and uninfected hosts can co-occur. Within these
268 populations, long term persistence of co-infections is possible, during which parasites
269 would probably be selected to collaborate. This possibility has rarely been explored to
270 date, although it has been found that in the parasitoid hymenopteran *Asobara tabida*
271 two facultative strains of *Wolbachia* have independent regulation but a third
272 obligatory strain may be influenced by the presence or absence of the other strains
273 (Mouton et al., 2004). *Spiroplasma* and *Wolbachia* probably are independently

274 regulated, as the *Spiroplasma* / *D. melanogaster* association is a very recent one. If
275 two strains have independent regulation, double infected hosts could suffer from a
276 higher fitness cost, as the number of bacteria would be much higher in double-
277 infected than in single-infected hosts, but we did not find such an effect.

278 Finally, our results are important for a better understanding of the dynamics of
279 *Wolbachia* and male-killing *Spiroplasma* in *D. melanogaster*. Facing inefficient
280 transmission (Montenegro et al., 2004) and with unknown or weak benefits arising
281 from their reproductive manipulation, it is not clear how *Wolbachia* and *Spiroplasma*
282 persist in *D. melanogaster* populations. One possible factor is the presence of other
283 direct fitness benefits, as observed for survival (Fry & Rand, 2004), or the lack of
284 deleterious effects, in which case, coupled with very high vertical transmission rate,
285 would demand only a small indirect benefit resulting from the reproductive
286 manipulation for the bacterium persist in a population. Our study, together with
287 existing data on temperature effects on transmission (Montenegro & Klaczko, 2004),
288 does not indicate the presence of these for *Spiroplasma*, at least in the host genetic
289 background upon which we conducted our tests. For *Wolbachia*, however, this could
290 be the case, as fitness benefits have been found (Fry & Rand, 2002; Fry, Palmer. &
291 Rand, 2004), and it causes a low level of cytoplasmic incompatibility (Reynolds &
292 Hoffmann, 2002).

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Table 1. Analysis of the larval competition experiment. Analysis of variance table on the rank transformed relative viability data, expressed as [wild type/(wild type +*spa*)] emerging adult individuals from each vial.

	df	Sum Sq	Mean Sq	*F*	*P*
Cytotype	3	0.01335	0.00445	0.4056	0.749
Density	1	0.00126	0.00126	0.1152	0.735
Cytotype x density	3	0.00699	0.00233	0.2126	0.888

Table 2. Analysis of female fecundity over 14 days. Wald's tests of the fixed effects on female fecundity, showing significance of maternal cytotype, density at which mother was raised as larvae and day of egg laying. Mother identity was entered in the generalized linear mixed model as a random factor. The significance reported is the change in deviance when the term is fitted last in the model.

	df	χ^2	P
Cytotype	3	4.84	0.184
Density	1	2.51	0.113
Day	13	1512.45	<0.001
Cytotype x density	3	2.23	0.526
Cytotype x day	39	32.06	0.777
Density x day	13	95.53	<0.001
Cytotype x density x day	39	42.92	0.307

Table 3. Adult female survival over 14 days. Analysis of deviance table measuring significance of cytotype and density at which adult female was raised as larvae on adult survival.

	df	Deviance	Residual df	Residual Deviance	*P*
Cytotype	3	0.51	557	1184.35	0.92
Density	1	48.72	556	1135.63	0.00
Cytotype x density	3	4.89	553	1130.74	0.18

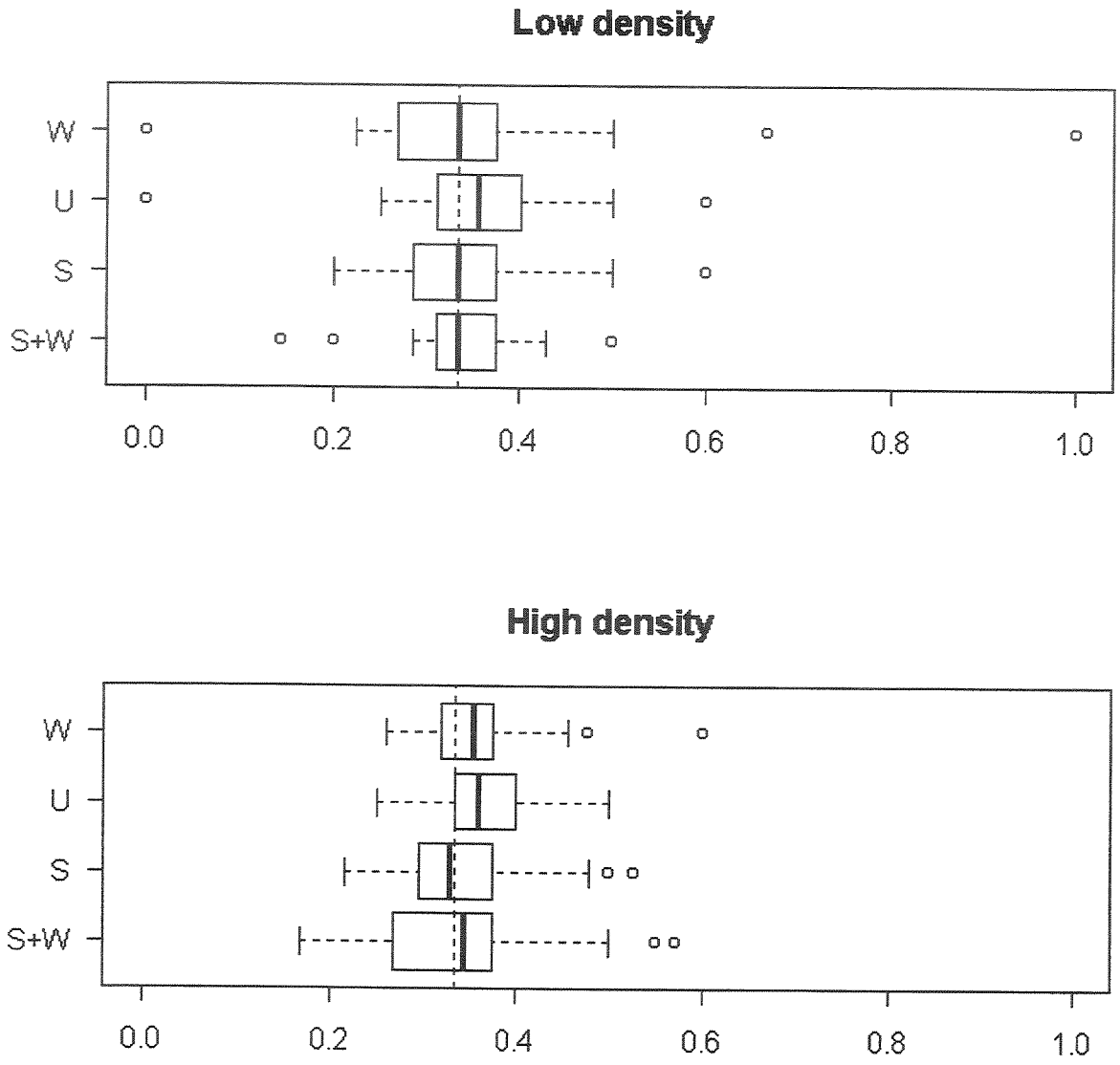

Figure 1. Boxplot (with median, 1st and 3rd quartiles, approximate 95% confidence intervals and outliers) of the relative ratio of emerging adults of larvae from the different cytotypes competing against *sparkling poliert* larvae. Upper plot, larvae grown at low density (nine larvae per vial); lower plot, larvae grown at high density (36 larvae per vial). W: *Wolbachia* cytotype, U: uninfected cytotype, S: *Spiroplasma* cytotype, S+W: double-infected cytotype. Vertical dashed line: expected relative ratio, equal to 1/3.

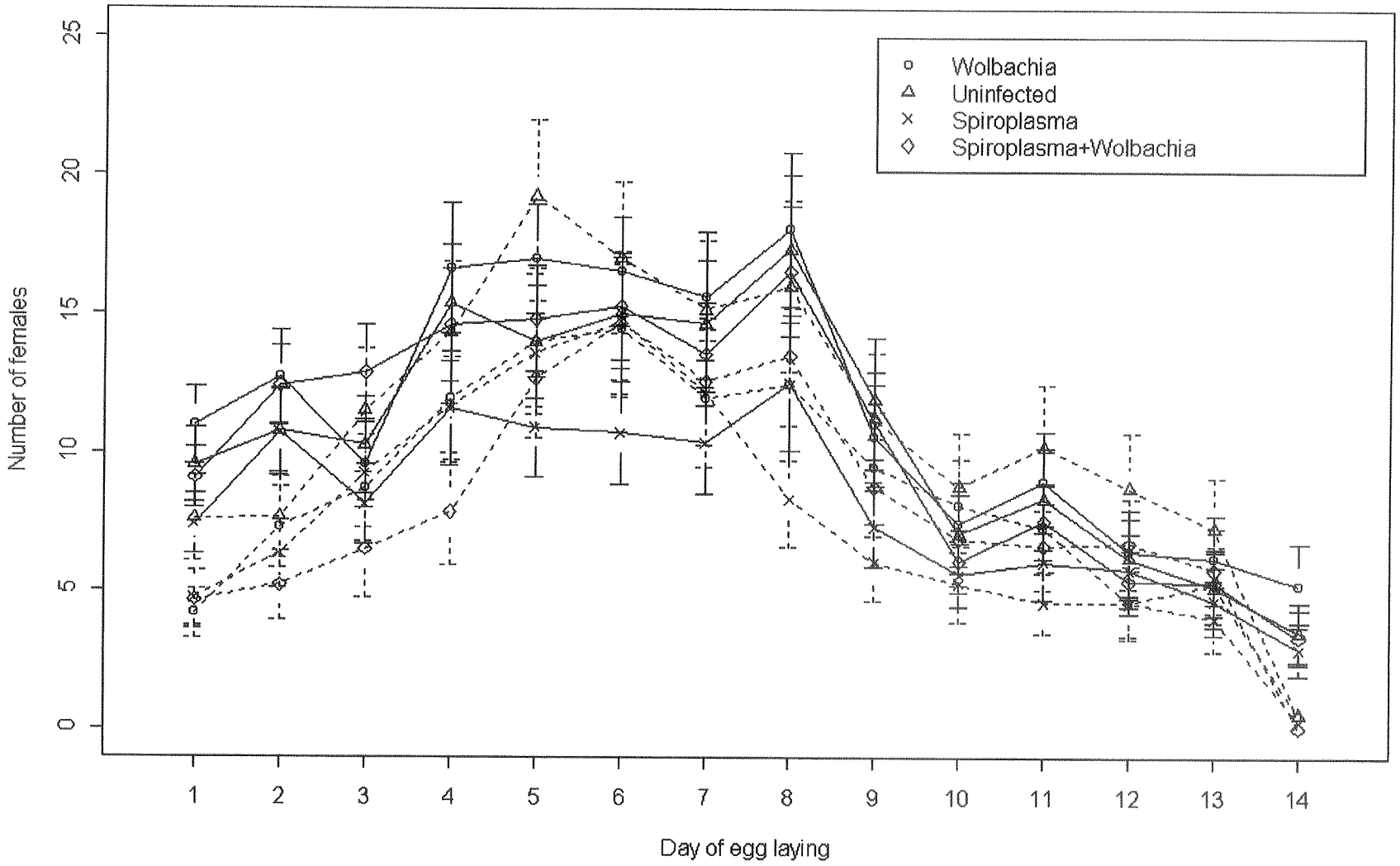

Figure 2. Daily means and standard errors of female progeny of each treatment from the female fecundity experiment. W=*Wolbachia*, U=uninfected, S=*Spiroplasma*, S+W=double-infected. Solid lines: low density, dashed lines: high density.

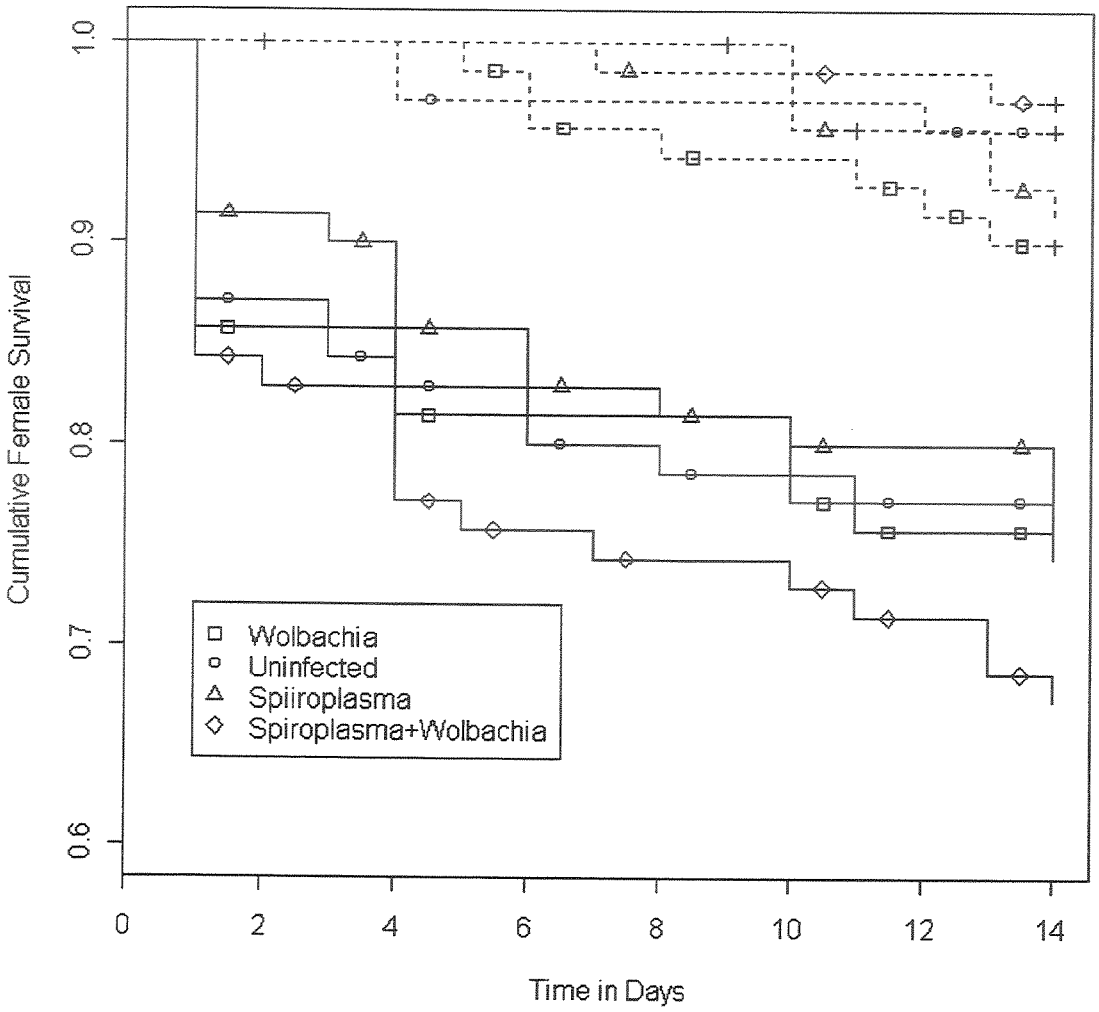

Figure 3. Plot of the cumulative survival over 14 days. Dashed lines: females grown at low density, solid lines: females grown at high density, crosshair: females that escaped or died as a result of human handling.

3.3) Artigo 3

Título:

Low temperature cure of a male killing agent in *Drosophila melanogaster*

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Short Communication

Low temperature cure of a male killing agent in *Drosophila melanogaster*

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Abstract

Environmental factors can affect transmission or phenotype expression of selfish cytoplasmic endosymbionts such as embryonic male killers. Temperature is one factor that usually affects the transmission rate of selfish cytoplasmic endosymbionts. Heat cures have been described for several host-parasite systems, cold cures, however, are rare. We report a temperature cure of the *Drosophila melanogaster* male-killing agent, which occurs when flies are raised at 16.5 °C. Flies grown at 20, 24, and 28 °C maintained an extremely female biased sexual proportion.

Bacteria and other endosymbionts that kill host male embryos (MK) are common among insects (Hurst and Majerus, 1993). They are one among several selfish cytoplasmic elements (SCE) that increase their own fitness inducing sex ratio deviations (SR) on their hosts (Cosmides and Tooby, 1981). Laboratory measures frequently overestimate SCE vertical transmission rates, because a number of environmental can affect transmission or phenotype expression (Weeks et al., 2002), resulting in discrepancies between field, and theoretical observations. Here, we report temperature effects on the transmission of a MK found in *Drosophila melanogaster* (Montenegro et al., 2000), which we are presently working to identify.

Host age also can be important for transmission and phenotype expression, and in MK two patterns of temporal variation have been observed. In the first pattern, transmission and male killing efficiency starts at a low rate and increases with female age (Ebbert, 1991). The second pattern is the opposite, killing efficiency and transmission have a high rate in young females, and decreases with female age (Brimacombe, 1980). Infected females of *D. melanogaster* show imperfect transmission when young and transmission approach perfection as the female ages (Montenegro et al., 2000). We also test if the temperature alters the temporal pattern of transmission of the MK agent in *D. melanogaster*, i.e., if some period of cold exposure is needed before MK transmission is clearly affected.

Females from the SR stock maintained at 24 °C were individually crossed with Canton-s males, transferred and raised for two generations at three different temperatures: 16.5, 20, or 24 °C. The flies were transferred to new vials two (24 °C), three (20 °C), or four (16.5 °C) times, respectively, as flies lay fewer eggs at lower temperatures. In each temperature treatment 15 females were allowed to produce offspring individually. For initiating the F2 generation we chose the offspring of three F1 females that had, in each temperature treatment, the most female biased broods. Five females of each of these three broods where taken from the last vial and crossed, thus the next generation was initiated with 15 females. Broods with more than 75% females were considered SR. We tried to test a higher temperature as well, but the high temperature group died due to an incubator malfunction. Thus we repeated the experiment with an additional 28 °C group. Both experiments had similar results: the MK was lost at 16.5 °C and progenies presented a 1♀:1♂ sex ratio, while other temperatures maintained MK transmission, and highly skewed sexual proportion (Table 1).

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Table 1
Sexual proportion at each temperature for the two generations

Temperature	F_1				F_2			
	♀	♂	% Males	% SR broods (n^*)	♀	♂	% Males	% SR broods (n^*)
First experiment								
16.5 °C	1005	4	0.4	100 (15)	439	374	46.0	0 (13)
20 °C	1201	1	0.1	100 (13)	1934	9	0.5	100 (14)
24 °C	1954	0	0	100 (15)	588	1	0.2	100 (12)
Second experiment								
16.5 °C	886	12	1.3	100 (14)	412	369	47.3	0 (13)
20 °C	2088	53	2.5	100 (15)	645	5	0.8	100 (11)
24 °C	1363	0	0	100 (14)	1632	5	0.3	100 (15)
28 °C	1436	0	0	100 (14)	1584	31	1.9	100 (15)

* Only progenies larger than 10 flies included.

In the second experiment we performed two extra sets of crosses using the F_1 females raised at 16.5 °C. To distinguish between phenotypic repression and transmission interruption of the MK, 15 females from the same three 16.5 °C broods, from the *last vial*, were crossed at 24 °C. To test if some period of cold exposure is needed before MK transmission is affected, 15 females from the same broods as the previous crosses, but from the *first vial*, were crossed at 16.5 °C. There were no differences among the three crosses, no SR progeny appeared (Table 2, $\chi^2 = 1.05$; df = 2; $P = 0.59$). Therefore transmission is interrupted also in early broods, and low temperature interrupts MK transmission, instead of just inhibiting its phenotypic effect. Cured strains were kept at room temperature for several generations, always showing 1♀:1♂ sexual proportion, indicating MK permanent loss.

With perfect transmission and no resistance genes, a MK agent will spread until it reaches fixation, driving host population to extinction. Temperature sensitivity of the MK agent can explain infected and non-infected females equilibrium in a population (Hurst et al., 2001). Also, in heterogeneous environments, different MK could be maintained in one population if they are susceptible to different temperature ranges. Several cases of heat cures have been reported (Hurst et al., 2001; Magni, 1953; Malogolowkin, 1959), less commonly cold cures (Kelly et al., 2002). Our results show another instance of low temperature SCE cure, suggesting that killing of the embryos may occur without transmission.

Table 2
Experimental conditions on the F_2 of females raised at 16.5 °C: (A) F_2 raised at 16.5 °C, offspring of females from last vial; (B) F_2 raised at 24 °C, offspring of females from last vial; (C) F_2 raised at 16.5 °C, offspring of females from first vial

Temperature	Vial	F_2♀	F_2♂	% Males	% SR broods (n^*)
(A) 16.5 °C	last	412	369	47.3	0 (13)
(B) 24 °C	last	674	627	48.2	0 (13)
(C) 16.5 °C	first	238	240	50.2	0 (9)

Chi-square contingency test. $\chi^2 = 1.05$, df = 2, $P = 0.59$.
* Only progenies larger than 10 flies included.

These results may also explain why *D. melanogaster* MK agent until now has only been found in the tropics, in warmer climates (Montenegro et al., 2000).

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4) Discussão e Conclusão Geral

Elementos genéticos egoístas de maneira geral são fenômenos comuns, e sua importância em evolução tem sido percebida de forma crescente. A importância teórica e prática que vem sendo atribuída a tais elementos, com o conseqüente investimento em pesquisa, faz com que novas perspectivas se abram e velhos problemas sejam encarados dentro deste quadro de pesquisa. As áreas mais diretamente beneficiadas pelo novo enfoque são a ecologia e a genética evolutiva (Hurst & Jiggins, 2000). Alguns dos exemplos de problemas em que **EGE** podem ter importância são: evolução da proporção sexual e mudanças nos sistemas de determinação de sexo (Werren, 1987; McVean & Hurst, 1996; Normark, 2004); envolvimento na especiação, como promotores de especiação ou como reforço no isolamento reprodutivo (Hurst & Schilthuizen, 1998); alterações nos padrões de seleção sexual (Randerson *et al.*, 2000); influência na organização do genoma de procariotos e eucariotos (Wu *et al.*, 2004, Hurst & Werren, 2001); e alteração do tamanho ótimo do número de ovos posto por apenas uma fêmea em um agregado (Hurst & McVean, 1998).

Endossimbiontes, sejam eles mutualistas obrigatórios ou parasitas reprodutivos, são criadores de novidades evolutivas e geradores de diversidade ecológica (Wernegreen, 2004). Mas a separação entre mutualista e parasita ou egoísta é muito mais tênue do que uma primeira análise permite entrever. Por exemplo, alguns parasitas reprodutivos podem aumentar a longenvidade de seus hospedeiros (Dobson *et al.*, 2004, Fry & Rand, 2002). Por outro lado, a transmissão de bactérias mutualistas está freqüentemente sob controle do hospedeiro – através de órgãos chamados bacteriossomos – possivelmente porque os hospedeiros foram selecionados para evitar que mutantes "egoístas" os manipulem (Normark, 2004). Outra observação condizente com esta hipótese é que o genoma de bactérias mutualistas obrigatórias é bastante reduzido e os genes

retidos são, principalmente, os que proporcionam novas vias metabólicas aos hospedeiros (Moran *et al.*, 2003; Wernegreen, 2004). Ou seja, o simbionte tem pouco "poder de manobra" sobre seu hospedeiro – embora uma explicação não mutuamente exclusiva é de que os simbiontes sofrem seleção para ter seu genoma reduzido, pois se replicarão mais rápido e terão maior transmissão do que variantes com mais genes, e os genes retidos são os que aumentam o valor adaptativo do hospedeiro. No entanto, enquanto as filogenias de simbiontes parasitas e seus hospedeiros são bastante incongruentes, as filogenias de simbiontes mutualistas e seus hospedeiros se sobrepõem perfeitamente (Wernegreen, 2004; Casiraghi *et al.*, 2001; Vavre *et al.*, 1999; Sauer *et al.*, 2000), possivelmente porque um endossimbionte parasita fatalmente acaba sendo eliminado da espécie hospedeira e a transmissão horizontal é importante para sua persistência a longo prazo.

O agente androcida de *D. melanogaster* é mais um exemplo de transmissão horizontal de um parasita reprodutivo. Ele é idêntico (pelo manos em 3000 pares de bases) ao de *D. nebulosa*, e ambos são bastante próximos de *Spiroplasma poulsonii*, o **AA** de *Drosophila willistoni* (Williamson *et al.*, 1999). A explicação mais parcimoniosa para esta distribuição é que o **AA** é ancestral no grupo *willistoni* e foi transferido para *D. melanogaster* recentemente, após a expansão de *D. melanogaster* para o Novo Mundo. A hipótese contrária, de que o **AA** foi de *D. melanogaster* para as moscas do grupo *willistoni*, envolve vários eventos de transmissão horizontal, o que é bastante improvável no curto espaço de tempo em que as espécies estão juntas. Finalmente, existe ainda a possibilidade de o agente androcida de *D. melanogaster* ter sido originado por interferência humana, em experimentos anteriores. Nestes experimentos, os **AA** de *D. willistoni*, *D. nebulosa, D. paulistorum* e *D. equinoxialis* foram transmitidos para *D. melanogaster* artificialmente, através de injeções de extratos de moscas infectadas em moscas saudáveis (Williamson & Poulson, 1979).

Quem dominará o conflito entre *D. melanogaster* e *Spiroplasma*? A persistência de um **AA** dentro de uma espécie representa uma pergunta ainda sem resposta, pois, além do hospedeiro sofrer forte seleção para resistência ao **AA**, as taxas de transmissão das fêmeas para suas filhas são sempre menores que 100% (Hurst, 1991). Não havendo transmissão horizontal ou algum aumento no valor adaptativo de fêmeas infectadas, apenas esta transmissão imperfeita é suficiente para que o **AA** desapareça (Hurst, 1991; Werren, 1987). Os parâmetros que estimamos sobre eficiência de transmissão (Montenegro *et al.* 2000; Montenegro & Klaczko, 2004) e a ausência de efeitos diretos do **AA** sobre o valor adaptativo em *D. melanogaster* sugerem que o destino do **AA** será, provavelmente, a extinção, a não ser que exista um aumento do valor adaptativo das fêmeas infectadas decorrente da morte dos machos, ou transmissão horizontal. Desta forma, existem três hipóteses para explicar a permanência de um **AA** numa população:

1. A morte dos machos proporciona transmissão horizontal para fêmeas não infectadas.
2. Diminuição do endocruzamento.
3. Realocação de recursos para as progênies infectadas.

A ecologia de alguns grupos é bastante consistente com uma ou mais destas hipóteses. Por exemplo, em besouros coccinelídeos (joaninhas predadoras de afídeos), as larvas recém-nascidas podem canibalizar os ovos de machos mortos. Uma larva que comeu um ovo antes de sair atrás de presas tem maior taxa de sobrevivência (Majerus & Hurst, 1997). Em *Nasonia vitripennis* (uma vespa parasitóide), além das fêmeas serem maiores e terem maior fertilidade com a diminuição da competição, em hospedeiros com alta densidade de vespas há transmissão horizontal para fêmeas não infectadas (Skinner, 1985). Jaenike *et al.* mostraram que, numa população subdividida com competição principalmente entre larvas aparentadas, um agente androcida pode subir de freqüência até fixar, causando a extinção da população. Contudo, este experimento envolvia uma quantidade grande de manipulação, e seus resultados são difíceis de

generalizar para poppulações naturais. De qualquer forma, para a maior parte dos agentes androcidas já descritos, faltam observações diretas sobre quais das três hipóteses propostas (ou, possivelmente, alguma outra) é a responsável por manter o **AA** na população.

Em *Drosophila*, apesar das freqüências do **AA** no campo serem relativamente baixas, em torno de 10% ou menores, elas são persistentes ao longo do tempo (Cavalcanti *et al.*, 1958; Ikeda, 1970; Williamson & Poulson, 1979). Porém, nenhuma das hipóteses parece ser muito consistente para explicar a persistência de um **AA** dentro das espécies do gênero (Hurst & Majerus, 1993). Criando-se moscas infectadas e normais juntas, nunca houve contaminação de moscas normais (Williamson & Poulson, 1979, Jaenike *et al.*, 2003). Mesmo em condições de alta densidade em populações de laboratório, que provavelmente favorecem a transmissão horizontal, as fêmeas **AA** diminuíram de freqüência (Ikeda, 1970; Ebbert, 1995). Apenas em uma ocasião (Carvalho & da Cruz, 1962), o **AA** de *D. willistoni* foi transmitido para moscas adultas normais de uma maneira menos artificial do que injeções, através de ingestão de macerados de moscas **AA**, no entanto, tentativas de repetir o experimento não tiveram sucesso (Williamson & Poulson, 1979). A própria lógica evolutiva de **AA** parece indicar que a transmissão horizontal não tem importância (Hurst, 1991). Apesar disto, Ebbert (1995) aponta que a transmissão horizontal é uma possibilidade largamente menosprezada e praticamente não explorada, nos casos de **AA** precoces.

Apesar de em geral espécies de *Drosophila* sofrerem depressão por endocruzamento, o grau de endocruzamento no campo é incerto e difícil de medir. Além desta dificuldade metodológica, existem outras características da história de vida de *D. melanogaster* que diminuem o endocruzamento. Por exemplo, as fêmeas de *D. melanogaster* emergem das pupas antes dos machos (Nunney, 1983), e as fêmeas amadurecem sexualmente entre dois e quatro dias após a emergência (Manning, 1967; Markow, 1996), quando iniciam o acasalamento. Já para avaliar a relevância da hipótese da diminuição da competição, é necessário saber se a competição

entre as larvas é significativa, e se ocorre entre larvas aparentadas. Estes aspectos da ecologia de *D. melanogaster* ainda são pouco estudados e incertos.

Em *Drosophila melanogaster*, um **AA** nunca havia sido descrito antes em populações naturais (Fitz-Earle & Sakaguchi, 1986; Hurst, 1993), e pensava-se que a espécie não seria favorável à invasão e manutenção de um **AA** (Hurst, 1993). Apesar de termos encontrado e descrito um **AA** em *D. melanogaster*, talvez esta última afirmação não seja de todo incorreta, pois apenas duas populações infectadas foram encontradas, e com o agente androcida em baixa freqüência. Quando se leva em conta que *D. melanogaster* é coletada mundialmente por dezenas ou centenas de laboratórios, é surpreendente que não se tenha encontrado antes este agente androcida, afinal, *D. melanogaster* é um dos principais organismo-modelo em biologia, inclusive da genética de populações (Rubin, 1988, Powell, 1997).

É difícil estimar a distribuição geográfica do *Spiroplasma* em *D. melanogaster*, uma vez que sua freqüência parece ser bastante baixa, em torno de 0~5%. Para um evento raro, mas de probabilidade desconhecida, o intervalo de confiança de 95% de uma amostra sem observações deste evento é dado por (aproximadamante) 3/n, onde n é o tamanho da amostra. Isto significa que, num local onde o **AA** não foi coletado, para se afirmar com 95% de confiança que o intervalo da freqüência do **AA** é entre 0-1%, precisa-se de um tamanho amostral de aproximadamente 300 fêmeas. Mesmo assim, o **AA** provavelmente está em equilíbrio em algumas populações; coletas em Recife encontraram novamente o **AA**, com freqüência similar à anteriores (5%; intervalo de confiança de 95%: 1,6-11,3%; n=100). Isto mostra que, apesar de recente e com baixas freqüências nas populações, o agente androcida de *Drosophila melanogaster* veio para ficar.

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6.1) Anexo 1

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Male-killing selfish cytoplasmic element causes sex-ratio distortion in *Drosophila melanogaster*

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Male-killing selfish cytoplasmic element causes sex-ratio distortion in *Drosophila melanogaster*

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Sex ratio distortion induced by a male-killing agent has been found to affect *Drosophila melanogaster*. The trait was discovered accidentally in a collection of flies from markets in Campinas, São Paulo State, Brazil. Repeated crosses with Canton-S males (for 15 generations to date) and successful transmission using the injection of macerates of sex ratio flies, have shown that the trait is inherited maternally, is cytoplasmic and is infectious. Crosses with strains marked with the visible mutation *white* and viability experiments at pre-adult stages of development, indicate that the skewed sex ratio results from male mortality before hatching. Males do not transmit the trait to their progeny.

Keywords: cytoplasmic inheritance, sex ratio, Spiroplasma, SRO.

Introduction

Selfish *genetic* elements (SGE) produce departures from Mendelian proportions and increase their own frequencies, often at the expense of host fitness (Werren *et al.*, 1988). Selfish *cytoplasmic* elements (SCE) are a special case of SGE. Since only females transmit SCE, they suffer strong selection to deviate the sex ratio or resource allocation towards females. This is usually accomplished through male sterility in plants (Saumitou-Laprade *et al.*, 1994) and other endosymbionts that alter the sex ratio away from 1♀:1♂, including male killers (Williamson & Poulson, 1979; Hurst, 1993).

Sometimes the progenies of females collected in the wild show an exceptional excess of females — a trait commonly called 'sex-ratio' (SR). The first SR cases analysed were determined paternally and produced by driving X chromosomes in *Drosophila obscura* (Gershenson, 1928) and *D. pseudoobscura* (Sturtevant & Dobzhansky, 1936). Maternally inherited SR traits were described later in several *Drosophila* species (for a review see Hurst, 1993). All were inherited cytoplasmically, as shown by the male killing (MK) at the embryonic stage. In one case, the SR trait was found to be infectious (Malogolowkin & Poulson, 1957). Howard (1942) reported an intriguing and complex picture in *Armadillium vulgare* that he ascribed to the meiotic drive of the sex chromosomes in females (Z or W drive), which produced broods with an excess of males and/or females. More recently, Juchault *et al.* (1992) explained the situation as two selfish sex-ratio factors, one cytoplasmic and the other one with predominantly, but not exclusive, maternal transmission. Cavalcanti *et al.* (1958) noted that the simultaneous action of cytoplasmic factors and nuclear modifiers could blur interpretation of the results. Thus, only after obtaining several homozygous strains in *D. prosaltans* were these authors able to explain the observed SR in this species as being a consequence of a cytoplasmic male-killer and a recessive nuclear gene which disrupts transmission of the cytoplasmic factor. Moreover, parthenogenesis can also produce all-female broods (Lanier & Oliver, 1966; Stouthamer & Werren, 1993). Thus, if one attempts to explain a newly observed SR in terms of male-killing SCE all other possible causes must be ruled out.

Hurst (1991) proposed a distinction between early and late MK, on the grounds that the evolutionary strategies underlying these two phenomena were completely different. Early male-killers have an advantage in that kin-selection favours clonal relatives of the suicide element located in females whereas with late MK there is a chance of horizontal transmission to uninfected females. In *Drosophila*, early MK agents have been reported in nine species: *D. bifasciata* (Magni, 1953), *D. prosaltans* (Cavalcanti *et al.*, 1958), *D. borealis* (Carson, 1956), *D. willistoni*, *D. paulistorum*, *D. equinoxialis*, *D. nebulosa*,

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D. robusta (Williamson & Poulson, 1979) and *D. roehrae* (Vaz *et al.*, 1998). Only in the *willistoni* group has the causative agent been identified, as a *Spiroplasma* (Class Mollicutes, Order Mycoplasmatales) (Hackett *et al.*, 1986).

So far no male-killing endosymbiont has been found in any natural population of *D. melanogaster* (Fitz-Earle & Sakaguchi, 1986; Hurst, 1993). We report here for the first time the occurrence of a sex-ratio trait in this species which is maternally inherited, infectious and caused by early MK.

Materials and methods

Collection, maintenance and selection of SR strain

In order to perform an experiment not related to sex ratio distortion, we collected adult flies in two markets in Campinas, São Paulo State, Brazil, on 16 July 1997. To set up isofemale lines, the females were placed individually in bottles with standard cornmeal medium, at room temperature. After 7 days, they were etherized to death and placed individually in wells of ELISA plates containing alcohol 70%. The progenies were counted after all adults had hatched. In orfer to distinguish *D. melanogaster* from its sibling species, *D. simulans*, the offspring were examined and the species separated according to the genital arch of the males. Forty-three lines were set up. Five did not produce offspring; 35 were found to be *D. simulans*; and only three were *D. melanogaster*, one of which — strain VFA-11 — had no males among more than 80 flies examined.

We took two samples of 13 flies each from this strain and crossed them to Canton-S males and to males from another strain (VFA-9). In both cases, no males appeared in the progenies. From then on, all experiments were performed using flies from the VFA-11 × Canton-S cross (hereafter called SR).

Initially, the stocks were maintained through mass transfer of SR females and the addition of Canton-S males. Nevertheless, after a few generations, the male frequency gradually rose from 0% to about 30%. At this point, we started a selection procedure to maintain the SR strain. In the first generation, 14 virgin SR flies were crossed individually to two Canton-S males, after which, at each generation, we chose five females from each of the four most fertile lines and with the most skewed sex ratios (20 females in total). These were crossed individually to two Canton-S males. Females from later broods of the selected lines were subsequently used to start a new generation. From the seventh generation onwards, the total number of selected females varied from 5 to 15, but always with five females per selected line.

Crosses to males with visible mutations

An altered sex ratio may have several aetiologies, including feminization of genetic males, parthenogenesis, male-killing or gametic selection. To rule out the first two hypotheses in the present case we crossed SR females with males from a *white* (recessive X-linked mutation) strain, and back-crossed the daughters to *white* males. If no *white* females appeared in the F_1 feminization was discarded. If the back-cross progeny showed the *white* mutation, parthenogenesis was excluded. However, these crosses cannot differentiate gametic selection from male-killing.

Egg–larva, larva–pupa and pupa–adult viability

The next step was to distinguish between gametic selection — male zygotes not being formed (primary sex ratio) — from the killing (early or late) of male zygotes (secondary sex ratio). To do so, we compared the mortality rates at various developmental stages of normal and SR strains. After collecting eggs and larvae, the egg–larva, larva–pupa and pupa–adult hatch rates were scored. Eggs were collected from slides covered with coloured medium on which the flies had been allowed to oviposit for up to 8 h, when slides were replaced. The eggs were picked just after slide removal, and were maintained at 24°C. Larvae and unhatched eggs were counted 30 h after egg collection. The larvae were collected as described for eggs, but the slides were changed every 24 h: the larvae were then collected after a further 24 h and transferred to small vials containing 10 mL of medium (100 mL distilled water, 4 g agar, 16 g dry yeast and 5 g sugar) with 10 larvae per vial, and maintained at 24°C. Pupae and adults were counted after at least 12 days; the adults were also sexed.

Demonstration of infectivity

Horizontal transfer is a test that can help to link an SR trait to an infectious agent (Ebbert, 1993). Because obligate endosymbionts are often fastidious to culture, thus hindering the fulfilment of Koch's postulates, this test is useful in revealing the nature of the trait at the outset of a study. We therefore used injections of macerated flies to determine whether an infectious agent was the cause of the observed sex ratio condition.

For this, we used a slight modification of the L'Heritier's (1958) method. In the first experiment, a macerate of 100 15–20-d-old SR flies was centrifuged in 1 mL of Waddington's modified *Drosophila* Ringer solution (solution A contains NaCl 37.5 g, $CaCl_2$ 1.0 g and, KCl 0.5 g in 500 mL of distilled water and solution B contains $NaHCO_3$ 1.0 g in 500 mL distilled water; the

solutions are autoclaved separately and mixed when needed in a ratio of one part of A, one part of B and eight parts of sterile distilled water) for 15 min at 3000–4500 r.p.m. In the second experiment, 150 flies were macerated. The supernatant, kept on ice, was injected into etherized 1–7-d-old virgin females, from normal Canton-S strains. As a control, a supernatant of Canton-S females, obtained in the same way, was injected into Canton-S females. The macerate was injected at the junction between the thorax and the scutelum. Each recipient fly received approximately 0.1 μL. Injected females were left to recover for one day in separate vials, after which two or three Canton-S males were added. The vials were changed at intervals ranging from 3 to 7 days, until the fly died or did not produce offspring.

Tests of males

The males that appeared in SR lines were crossed to Canton-S females to establish whether males transmit the sex ratio condition to their broods. We used two controls. In the positive control, SR females, sisters of the males being tested, were crossed to Canton-S males. In the negative control (normal sex ratio), Canton-S females were crossed to Canton-S males. In a few cases, in addition to SR females from the same brood in which males appeared, SR females from a later brood were tested. All crosses were performed as single-pair matings so that the progenies could be scored as single female productions. Pairs were put into small vials with approximately 20 mL of cornmeal medium and were moved to new vials at variable intervals, but always allowing the scoring of at least three broods. If the progeny had a sex ratio of 3 females: 1 male (or more skewed) it was considered as SR progeny (Sakaguchi & Poulson, 1963). If the progeny started with an even sex ratio and later showed an SR ratio (as defined above), it was considered to be a progressive SR progeny. The remaining cases were considered normal progenies.

Results

Maintenance and selection of SR strain

In order to maintain SR, we carried out a selection procedure for 15 generations, back-crossing the females to Canton-S males at each generation. This resulted in a strain with more than 99.9% of the Canton-S genetic background. Table 1 shows the total progeny in each generation, along with the number of producing females and SR females per generation. To date, 14 571 flies have been counted, with 14 463 females and only 108 males (0.7%). Among 154 individual progenies scored, only one had a normal (1♀:1♂) sex ratio. In addition to the normal strain, males also appeared in 11 SR lines, with the highest male frequency being 15% (36♀, 4♂), always in the first broods (males were absent in later broods). This trend was also observed in other instances in which males appeared.

Crosses to males with visible mutations

Overall, the results from the crosses with *white* males indicated that fertilization was occurring, whereas feminization was not. In the first generation of SR females individually crossed to *white* males, there was neither a single white fly nor a male in the progeny

Table 1 Results from the selection procedure used for sex ratio maintenance

Generation	♀ Offspring produced	♂ Offspring produced	♀♀ Producing SR progenies	♀♀ Producing normal progenies
1	492	0	8	0
2	1569	8	18	0
3	3057	0	18	0
4	3384	4	20	0
5	1302	0	17	0
6	937	1	16	0
7	653	0	14	0
8	832	0	14	0
9	150	0	5	0
10	292	10	5	0
11	605	0	5	0
12	52	0	2	0
13	756	0	5	0
14	160	2	4	0
15	222	83	3	1

Table 2 Results of crosses of SR females to males from a *white* strain. In the first generation eight females of the VFA-11 strain (SR) were crossed to *white* males. From the F_1, eight females were backcrossed to *white* males

F_1	Backcross
♀ VFA-11 × ♂ white	♀ (♀ VFA-11 × ♂ white) × ♂ white
170 ♀ +	400 ♀ + , 425 ♀ white

(Table 2, summarizes the results for 170 flies, with pooling of the progenies). If feminization occurred, one would expect some of the females to be *white*. In the second generation — backcross to *white* males — 825 flies were examined. All were females, with 400 being wild type and 425 *white*, in agreement with the expected 1:1 Mendelian ratio among females. This unequivocally shows that fertilization occurred and that the sex-ratio trait observed is not a result of parthenogenesis.

Viability experiments

The egg–larva viability in the SR strain was half (41.2%) that of the normal strain (82.7%) (Table 3). However, the larva–pupa and pupa–adult viabilities were the same in the two strains (Table 4). The larva–adult viability in the normal strain was 92.8%, while in the SR strain it was 94.5%. These results show that male embryos are dying very early in the development and this is probably the cause of the sex-ratio deviation. Moreover, females seem to be little — or not at all — affected by the killing agent.

Injection experiments

Initially, the injection experiments did not yield encouraging results at first sight. In the first experiment, not a single control fly produced sufficient offspring for comparison with SR injected flies. None of the 13 females that produced enough progeny had overall SR broods. Only one at the very end of its life produced SR broods (a progressive SR fly). The total production did not differ significantly from a 1♀:1♂ sex ratio (780♀, 714♂, $\chi^2 = 2.92$, $P = 0.09$). Because no control remained, only a few tests were performed to account for transmissions to subsequent generations. Nevertheless, two injected females — one of them being the one mentioned above — gave origin to lines that eventually became SR. One of the strains was maintained without producing males (more than 98% females) for five generations, after which it was lost. The other has been maintained by the same procedure used in the selection experiment. Currently, this line is in the ninth generation, with skewed progenies (more than 99% females).

Table 3 Egg–larva viability in normal (Canton-S) and SR strains

Strain	Eggs counted	Larvae hatched (% of total)
SR	153	63 (41.2%)
Canton-S	162	134 (82.7%)

Table 4 Larva–pupa and pupa–adult viability in normal and SR strains

Strain	Larvae tested	Pupae formed (% of larvae)	Adults hatched (% of pupae)
SR	90	87 (96.7%)	84♀ and 1♂ (97.7%)
Canton-S	140	131 (93.6%)	72♀ and 58♂ (99.2%)

In the second experiment all of the lines injected with SR macerated flies and their controls produced normal progenies, except one female in the experimental group. It started with a normal sex ratio and developed into a skewed one [the consecutive counts were 73(♀):63(♂); 64:52; 63:17; 2:0]. In the second generation we tested 47 females of the experimental group and 30 from the controls (data not shown). Only one female — from the experimental group — was SR (36♀:2♂). Using this fly progeny, we established an SR strain that has been kept for eight generations with a consistently skewed sex-ratio (more than 92% females).

Tests of males

The crosses with males that appeared in SR lines vs. Canton-S females did not produce any SR line, and were not significantly different ($\chi^2 = 1.52$, $P = 0.22$) from the sex ratio in the control lines of Canton-S females × Canton-S males. In contrast, 72% of the crosses involving SR females produced SR or progressive SR progenies (Table 5). This shows that only females transmit the SR condition to their progenies.

Discussion

During a collection at markets in Campinas, São Paulo State, Brazil, we found a *D. melanogaster* female that produced highly skewed progeny with an excess of females. This distorted sex ratio has been maintained for 15 generations by outcrossing SR females to males of a normal laboratory strain, showing that the trait is maternally inherited. The maternal inheritance and the transfer of SR by the injection of macerates of SR flies into Canton-S normal flies shows that a selfish cytoplasmic element caused the SR. The results of crosses to

Table 5 Results of male transmission tests (ratios expressed in ♀:♂)

	SR ♀	Progressive SR ♀	Normal ♀
♀ Canton-S × ♂ Canton-S	0	0	21 (3226:3078)
♀ Canton-S × ♂ SR	0	0	24 (3127:3118)
♀ SR × ♂ Canton-S	36 (5558:19)	5 (809:371)	16 (2595:2392)

white males, together with the fact that the hatching rate of SR lines was half of the normal lines, strongly suggests that SR distortion resulted from the early death of male embryos. The present case shares some features with other reports of MK instances: incomplete transmission, occurrence of progressive SR females, which is rarer than reversion to normal females or full transmission (Ebbert, 1993), and early broods, which are more prone to contain males and uninfected females (Ebbert, 1991).

The present finding raises up to 10 the number of *Drosophila* species known to be affected by MK agents. It is possible that other *Drosophila* species may bear them. However, the persistence of MK agents within drosophilids has not yet been well explained. Data are still lacking to support the supposed advantages of resource reallocation, avoidance of inbreeding and horizontal transmission that could result from killing males and compensate incomplete symbiont transmission (Ebbert, 1993; Hurst, 1993).

The nature of the MK agent described here remains to be investigated. *D. melanogaster* was the recipient species at various MK transfer experiments involving spiroplasmas that infect the *willistoni* group (review in Williamson & Poulson, 1979). Whether any such transfers later resulted in a spread to natural populations, has not been investigated. It is tempting to speculate that the androcidal agent of *D. melanogaster* is an escape from any of these experiments.

Several questions arise from the finding of the *D. melanogaster* MK agent. Are the androcidal agents in *D. melanogaster* and in the *D. willistoni* group species the same? If so, was it a natural transmission? Or, is this finding a consequence of the experiments of Poulson and coworkers in the early MK studies? Will the element persist in the population, or will it go extinct?

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