Increased fecundity associated with infection by a *Cytophaga*-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*

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The endosymbiont *Wolbachia* has gained widespread notoriety over the past decade because of its high infection frequency among arthropods, and the unique heterogeneity of the host reproductive effects that it has been implicated as causing to enhance its own spread. Recently, another endosymbiotic bacterium from the *Cytophaga*–*Flavobacterium*–*Bacteroides* phylum has been shown to be widespread among arthropods and manipulate its hosts' reproduction to enhance its own spread. We show that infection by this *Cytophaga*-like organism (CLO) in the predatory mite *Metaseiulus occidentalis* (Acari: Phytoseiidae) is associated with a significant increase in the fecundity of infected females. This adds to the growing list of phenotypes that the CLO can induce in its hosts, which now include feminization, parthenogenesis induction, cytoplasmic incompatibility and fecundity enhancement, rivaling *Wolbachia* for overall diversity of host reproductive manipulations.

Keywords: *Cytophaga*-like organism; *Wolbachia*; *Metaseiulus occidentalis*; increased fecundity; host effects

1. INTRODUCTION

Maternally inherited endosymbionts spread through populations by increasing the relative fitness of infected females. This can be achieved by their increasing the fecundity and/or survival of infected females relative to uninfected females through metabolic processes (Douglas 1994) and is therefore beneficial to both host and symbiont (in a mutualistic association). However, another class of endosymbionts are more parasitic in nature and manipulate the reproductive systems of their hosts to enhance their own spread, inducing phenotypes known as parthenogenesis induction, feminization, male-killing and cytoplasmic incompatibility. The best known of these reproductive parasites is the α -proteobacterium *Wolbachia*, because of its extremely high incidence among arthropods and the unique heterogeneity of the host reproductive manipulations and related phenomena they can induce (Stouthamer *et al.* 1999).

Recently, an intracellular bacterium from the *Cytophaga*–*Flavobacterium*–*Bacteroides* phylum has been implicated as causing feminization in a species of mite (Weeks *et al.* 2001), cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella* (Hunter *et al.* 2003) and associated with parthenogenesis in several other species from the *Encarsia* genus (Zchori-Fein *et al.* 2001). A recent screening study (Weeks *et al.* 2003) has also shown that this *Cytophaga*-like organism (CLO) is prevalent among arthropods, and that double infections of the CLO and *Wolbachia* can occur within single individuals of a species. We examine the effects of infection by the CLO in the parahaploid predatory mite *Metaseiulus* (= *Galendromus* = *Typhlodromus*) *occidentalis* (Nesbitt).

2. MATERIAL AND METHODS

A sample of 50 adult *M. occidentalis*, obtained from the biocontrol company Biotactics Inc. (Perris, CA), was used to start a base population. Mites were reared on detached leaves of the common bean (*Phaseolus vulgaris*) on cotton wool soaked in water in a climate chamber at 25 °C, 50–70% relative humidity and under 24 h daylight. Mites were fed on a mixture of eggs, immatures and adults of the carmine spider mite, *Tetranychus cinnabarinus* (which was not infected with either *Wolbachia* or the CLO). Samples of 20 F₂ females taken from the base population were all infected with the CLO, but none was infected with *Wolbachia* (samples were screened using the hemi-nested PCR method of Weeks *et al.* (2003)).

To control for nuclear effects induced by the tetracycline treatment and any subsequent drift effects between tetracycline-cured and uncured lines, we used inbred lines for the crossing experiments. Three inbred isofemale lines were created by brother–sister matings within each line for four generations, resulting in an expected inbreeding coefficient of 0.59 (Li 1955). From each inbred isofemale line we then created a CLO-uninfected counterpart by injecting (using glass needles) tetracycline hydrochloride dissolved in water (0.15%) into adult carmine spider mites and feeding them to starved virgin female and male *M. occidentalis* from each inbred line. All F₁ progeny/inbred lines were then tested for infection by PCR and found to be uninfected.

The effects of the CLO infection on host reproduction in *M. occidentalis* were determined by crossing experiments between the CLO-infected and uninfected sub-lines within each of the inbred lines. Sixty inseminated females from each sub-line/inbred line (generation 4 after tetracycline treatment) were isolated on bean leaves. After 24 h, eggs were isolated individually on bean leaf discs (2 cm diameter) on water-soaked cotton wool, infested with carmine spider mites, and left to develop until they could be sexed. Single pairs of 1–2-day-old adult virgin females (F) and males (M) were then set up on bean leaf discs in all possible crosses between infected (I) and uninfected (U) individuals within inbred lines (i.e. F × M: I × I, I × U, U × I, U × U). After 24 h, males were removed (stored at –80 °C) and females were transferred daily for 5 days to fresh bean leaf discs containing all stages of spider mites as prey. After 6 days, female parents were removed from the last leaf disc and stored at –80 °C. For inbred line 1, the number of eggs (fecundity) and egg viability were recorded for each leaf disc (day). For inbred lines 2 and 3, the number of eggs, egg viability, F₁ mortality (egg-to-adult mortality) and the adult sex ratio were recorded for each leaf disc (day). After the crossing experiments were complete, all parental females and males were individually screened by PCR for the CLO to confirm infection status.

In all inbred line crossing experiments, some or all variables were not normally distributed and/or variances were heteroscedastic. Normality and/or homoscedasticity did not improve by transformation. We used univariate ANOVAs to determine *F*-ratios for each variable with cross type as a factor, and then determined the significance of the *F*-ratio through permutation tests using the Monte Carlo simulation procedure (10³ permutations). Permutation tests give exact significance, are free from assumptions about distributions and variances and have more power than non-parametric methods (Good 1994). Variables for which a significant effect of cross type was detected were further investigated by pairwise comparisons using *t*-tests, with significance again determined by permutation tests using Monte Carlo simulation (10³ permutations). All *p*-values were corrected for multiple comparisons using the Dunn–Sidak method (Sokal & Rohlf 1995).

Table 1. Mean fecundity and egg viability over 6 days for crosses between CLO-infected (I) and uninfected (U) *Metaseiulus occidentalis* from inbred line 1 (L1).

(Permutation tests (10^3 permutations) were used to determine significance for univariate ANOVAs (for each variable, with cross type as a factor) and *t*-tests (pairwise comparisons between factors when a variable was significant). Different letter superscripts (a, b) within a column represent significant differences (by *t*-tests) between means at the $\alpha = 0.05$ level after corrections for multiple comparisons (whilst the same letter indicates non-significance). *** $p < 0.001$; M, male; F, female.)

cross (F $\times$ M)	<i>n</i>	fecundity $\pm$ s.e.***	egg viability $\pm$ s.e.
I $\times$ I	17	11.235 $\pm$ 0.609 ^a	0.870 $\pm$ 0.021
I $\times$ U	20	10.8 $\pm$ 0.408 ^a	0.916 $\pm$ 0.017
U $\times$ I	21	6.095 $\pm$ 0.621 ^b	0.880 $\pm$ 0.032
U $\times$ U	12	8.25 $\pm$ 0.818 ^b	0.901 $\pm$ 0.030

3. RESULTS

The results of reciprocal crosses between the CLO-infected and uninfected mites from three different inbred lines are shown in tables 1–3. For inbred line 1 (L1), there was a significant effect of cross type on fecundity ($F_{3,66} = 17.71$, $p < 0.001$), but not on egg viability (table 1). Pairwise comparisons for fecundity showed significant differences between crosses involving infected females (I $\times$ I, I $\times$ U) and those involving uninfected females (U $\times$ I, U $\times$ U) after correcting for multiple comparisons. Infected females always produced significantly more progeny than uninfected females, regardless of the infection status of males. By permutation tests, uninfected females mated to infected males (U $\times$ I) produced significantly fewer offspring than uninfected females mated to uninfected males (U $\times$ U) ($t = 1.99$, d.f. = 31, $p = 0.045$), but this was not significant after correcting for multiple comparisons.

Inbred line 2 (L2) showed a significant effect of cross type on fecundity ($F_{3,101} = 19.38$, $p < 0.001$; table 2). There was no significant effect of cross type on egg viability, F_1 mortality or sex ratio (proportion of males). Pairwise comparisons between cross types for fecundity showed significantly increased fecundity for females infected with the CLO compared with females uninfected with the CLO. In addition, uninfected females mated to infected males produced fewer offspring than uninfected females mated to uninfected males ($t = 2.51$, d.f. = 61, $p = 0.008$), although significance was marginal after correcting for multiple comparisons. Mortality (at both egg and pre-adult stages) was high in all crosses for this inbred line, perhaps as a result of the fixation of deleterious alleles during inbreeding.

The last inbred line (L3) produced similar results to the previous crosses. There was a significant effect of cross type on fecundity ($F_{3,86} = 29.37$, $p < 0.001$) and sex ratio ($F_{3,85} = 4.58$, $p = 0.004$), but not on egg viability or F_1 mortality (table 3). Pairwise comparisons between cross types for fecundity again showed significantly increased fecundity for females infected with the CLO compared with uninfected females. Uninfected females mated to infected males (U $\times$ I) produced significantly fewer offspring than uninfected females mated to uninfected males

($t = 3.08$, d.f. = 44, $p = 0.001$). Pairwise comparisons between cross types for sex ratio showed that infected females mated to infected males produced a significantly less male-biased sex ratio compared with uninfected females mated to infected males ($t = 3.23$, d.f. = 44, $p = 0.0006$).

4. DISCUSSION

The crossing results from the three inbred lines showed a clear and significant increase in fecundity associated with infection by the CLO. The fecundity advantage of infected females was approximately 1.6 times that of uninfected females over a 6-day laying period. There was no elevated mortality in uninfected females, which is often found in mutualistic associations (Hoerauf *et al.* 1999). The increased fecundity associated with infection by the CLO in *M. occidentalis* is a new reproductive phenotype for the CLO. *Wolbachia* has been shown previously to cause positive fecundity effects (Stolk & Stouthamer 1996; Vavre *et al.* 1999; Dobson *et al.* 2002), although some of these studies did not control for nuclear background effects (Bordenstein & Werren 2000). We used four generations of brother–sister matings in our lines to control for any nuclear effects caused by tetracycline treatment in this study.

For all three inbred line crosses, uninfected females mated to infected males were less fecund than uninfected females mated to uninfected males. However, this trend was not significant for all inbred lines. Using the combined probability test (Sokal & Rohlf 1995), the trend is highly significant overall ($\chi^2 = 29.07$, d.f. = 6, $p = 0.0006$). The reason for this difference is not clear. The phenotype is analogous to cytoplasmic incompatibility; however, there is no elevated mortality or increased male production normally associated with cytoplasmic incompatibility in haplodiploid species (Vala *et al.* 2002). Further experiments (including backcrossing after curing) are required to determine whether this is a real reproductive manipulation or simply an artefact from our lines.

Johanowicz & Hoy (1998) have previously implicated *Wolbachia* as causing cytoplasmic incompatibility in their laboratory lines of *M. occidentalis*. However, Hess & Hoy (1982) had previously shown that another unidentified endosymbiotic bacterium was also present in their laboratory lines. Weeks & Breeuwer (2003) and Weeks *et al.* (2003) have since identified that bacterium as being the CLO. Johanowicz & Hoy (1998) cured *Wolbachia* infections in their laboratory lines of *M. occidentalis* by heat treatment at 33 °C for at least eight generations. The cytoplasmic incompatibility that they found in the incompatible cross (uninfected female mated to infected male) resulted in a decrease in fecundity, a reduced egg hatch rate with a high proportion of shrivelled eggs and the surviving progeny being all male. The decrease in fecundity is similar to what we found here, although they did not find an increase in the fecundity of infected females. It is difficult to draw any conclusions on their study based on our results. While it is probable that their lines contained the CLO, it is not known whether the heat treatment that they used to cure *Wolbachia* would also cure the CLO. More importantly, the effects on *M. occidentalis* from interactions between the CLO and *Wolbachia* cannot be predicted. Until these effects can be partitioned out, the

Table 2. Mean fecundity, egg viability, F_1 mortality (frequency) and sex ratio (proportion of males) over 6 days for crosses between CLO-infected (I) and uninfected (U) *Metaseiulus occidentalis* from inbred line 2 (L2).

(Permutation tests (10^3 permutations) were used to determine significance for univariate ANOVAs (for each variable, with cross type as a factor) and *t*-tests (pairwise comparisons between factors when a variable was significant). Different letter superscripts (a, b or c) within a column represent significant differences (by *t*-tests) between means at the $\alpha = 0.05$ level after corrections for multiple comparisons (whilst the same letter indicates non-significance). *** $p < 0.001$. M, male; F, female.)

cross (F × M)	<i>n</i>	fecundity ± s.e.***	egg viability ± s.e.	F_1 mortality (frequency) ± s.e.	proportion of males ± s.e.
I × I	23	10.043 ± 0.687 ^a	0.701 ± 0.042	0.478 ± 0.052	0.442 ± 0.043
I × U	19	9.947 ± 0.687 ^a	0.692 ± 0.072	0.499 ± 0.064	0.478 ± 0.058
U × I	34	5.676 ± 0.395 ^b	0.722 ± 0.038	0.484 ± 0.047	0.562 ± 0.066
U × U	29	7.276 ± 0.468 ^c	0.728 ± 0.048	0.494 ± 0.056	0.492 ± 0.051

Table 3. Mean fecundity, egg viability, F_1 mortality (frequency) and sex ratio (proportion of males) over 6 days for crosses between CLO-infected (I) and uninfected (U) *Metaseiulus occidentalis* from inbred line 3 (L3).

(Permutation tests (10^3 permutations) were used to determine significance for univariate ANOVAs (for each variable, with cross type as a factor) and *t*-tests (pairwise comparisons between factors when a variable was significant). Different letter superscripts (a, b or c) within a column represent significant differences (by *t*-tests) between means at the $\alpha = 0.05$ level after corrections for multiple comparisons (whilst the same letter indicates non-significance). ** $p < 0.01$; *** $p < 0.001$. M, male; F, female.)

cross (F × M)	<i>n</i>	fecundity ± s.e.***	egg viability ± s.e.	F_1 mortality (frequency) ± s.e.	proportion of males ± s.e.**
I × I	24	11.917 ± 0.477 ^a	0.949 ± 0.018	0.257 ± 0.027	0.403 ± 0.025 ^a
I × U	20	10.500 ± 0.473 ^a	0.931 ± 0.026	0.317 ± 0.038	0.411 ± 0.041 ^{a,b}
U × I	21	6.095 ± 0.402 ^b	0.908 ± 0.035	0.312 ± 0.052	0.595 ± 0.053 ^b
U × U	25	8.36 ± 0.486 ^c	0.944 ± 0.017	0.266 ± 0.029	0.470 ± 0.033 ^{a,b}

actual phenotypic effects of *Wolbachia* infection in *M. occidentalis* remain unknown. Further studies should focus on interactions between the CLO and *Wolbachia* in *M. occidentalis*. This is especially important given that Weeks *et al.* (2003) have found several arthropod species that harbour double infections of the CLO and *Wolbachia* in single individuals.

Finally, research into endosymbionts that manipulate their host's reproduction to enhance their own spread has been dominated by research on *Wolbachia*. The CLO has now been implicated as inducing a similar variety of reproductive phenotypes within their hosts that rivals *Wolbachia* for overall diversity (Weeks *et al.* 2001; Zchori-Fein *et al.* 2001; Hunter *et al.* 2003; this study). The screening study of Weeks *et al.* (2003) also shows that the CLO infects a significant number of arthropod hosts. It is probable that other endosymbionts can also cause similar reproductive manipulations; they just need to be found.

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