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Mushrooms as potential sources of *Wolbachia*-curing antibiotics

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ABSTRACT

Background: *Drosophila innubila* is infected with maternally transmitted male-killing *Wolbachia*. The mushrooms on which mycophagous *Drosophila* feed are chemically diverse. It is possible that some species contain antibiotics that could cure flies of their *Wolbachia* infections. Such curing would result in an immediate doubling of offspring production, would affect the prevalence of infection in natural populations, and would influence where *Drosophila* prefer to oviposit.

Methods: *Drosophila innubila* were reared on ~100 diverse species of mushrooms and their subsequent offspring sex ratios monitored for evidence of antibiotic curing of *Wolbachia* infection.

Results: None of the mushrooms tested provided compelling evidence of antibiotic activity against *Wolbachia*.

Keywords: antimicrobial, *Drosophila innubila*, endosymbionts, male killing.

INTRODUCTION

Wolbachia are endosymbiotic α-proteobacteria that infect innumerable species of insects. The spread of *Wolbachia* Hertig within their host species is accomplished by various means of reproductive manipulation, such as male killing and cytoplasmic incompatibility (Werren, 1997). An important determinant of the dynamics of these infections within populations is the fidelity of maternal transmission (Hurst, 1991; Turelli, 1994). In theory, perfect transmission of a male-killing endosymbiont could result in its spread to effective fixation and consequent extinction of the host species. Thus, incomplete transmission may be essential for the long-term persistence of associations between insects and maternally transmitted male-killers.

The cause of incomplete transmission can have a major effect on the dynamics of infection prevalence. On the one hand, if this is purely a random process that affects all females equally, then the fidelity of maternal transmission at the population level is effectively a constant in terms of its effect on infection prevalence. Alternatively, if

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incomplete transmission results from specific environmental factors, then conditions that vary spatially or temporally may affect the fidelity of maternal transmission and thus the population dynamics of the infection. In practice, incomplete transmission can result from failure of the bacteria to colonize an egg prior to oviposition or subsequent loss of the infection during offspring development, either of which might be susceptible to environmental conditions.

Wolbachia infections in laboratory cultures of insects are routinely cured by feeding antibiotics (e.g. tetracycline) to adults or rearing offspring on medium that contains antibiotics. It has therefore been suggested that some food resources in the wild may contain naturally occurring antibiotics that could cure insects of their *Wolbachia* infections (Clancy and Hoffmann, 1998; Dyer *et al.*, 2005). Given their chemical diversity, mushrooms would appear especially promising sources of such antibiotics. In fact, surveys have revealed that a substantial proportion of mushroom species exhibit antimicrobial activity, as revealed by *in vitro* assays showing inhibition of bacterial growth (Suay *et al.*, 2000; Rosa *et al.*, 2003). Thus, it is possible that insects feeding on certain species of mushrooms may become cured of their *Wolbachia* infections. Although *Wolbachia* cannot be cultured *in vitro*, the presence or absence of the infection can be readily assayed in insects carrying male-killing *Wolbachia*: infected females produce only daughters, while uninfected females produce normal offspring sex ratios (Hurst and Jiggins, 2000).

Here we report a survey of naturally occurring mushrooms for their ability to cure *Drosophila innubila* of their male-killing *Wolbachia*. *Drosophila innubila* is a mycophagous member of the quinaria group of *Drosophila* that occurs in woodlands and forests of the sky islands in Arizona and New Mexico. Between 10% and 40% of the females in the wild are infected with *Wolbachia*, and these females produce ~100% female offspring (Dyer and Jaenike, 2004, 2005). A single generation of larval development on medium containing the antibiotics tetracycline or rifampicin results in complete curing of the infection and subsequent production of normal 1 : 1 offspring sex ratios (Dyer *et al.*, 2005).

Because male killing in *D. innubila* occurs in the embryonic stage before the larvae have had a chance to feed (Dyer *et al.*, 2005), the F1 offspring of *Wolbachia*-infected females will consist solely of females, even if the mushroom on which they develop contains antibiotics. However, if the mushroom cures the F1 females of the *Wolbachia* infection, then the F2 generation will have a normal 1 : 1 sex ratio. Even partial curing of the infection, resulting in a reduced *Wolbachia* density within flies, leads to the production of significant numbers of male offspring (Dyer *et al.*, 2005). Thus, quantifying the sex ratio of the F2 generation is a potentially powerful, yet simple method to detect the existence of fungal antibiotics to which *Wolbachia* are sensitive.

MATERIALS AND METHODS

The *D. innubila* used in these studies were collected in September 2000 in the Chiricahua Mountains, Arizona. The *Wolbachia*-infected strain *mk-3* had been maintained by crossing females of this strain with males of the uninfected strain *ST-1*. Because *Wolbachia* are maternally inherited, these crosses result in ~100% female offspring. A single generation of development on medium containing either tetracycline or rifampicin cures *mk-3* females of *Wolbachia*, resulting in their production of normal offspring sex ratios.

Mushrooms were collected during forays conducted by the Rochester Area Mycological Association at Letchworth State Park, in Wyoming and Livingston Counties, New York.

The collections were made on 28 September 2003 and 29 September 2004. The specimens were identified on the basis of macroscopic characteristics by expert mycologists, and voucher specimens kept in case any tested positive for antibiotic activity. The ~100 species of mushrooms tested included both *Ascomycetes* and *Basidiomycetes*, including a variety of polypores, boletes, and a wide variety of gilled mushrooms. The mushrooms were kept fresh (not frozen or dried) and set up for testing within 24 h of collection. A sample mushroom of each species was cut into three pieces, which were placed into three replicate vials on top of moistened cotton. Because no other food was available, larval food consumption was limited to the test mushroom.

Mated females of *D. innubila* the *Wolbachia*-infected strain *mk-3* were placed individually in the vials and allowed to feed and oviposit for 10 days. Upon emergence, F1 adults were transferred to standard laboratory medium, which consists of Instant Drosophila Medium (Carolina Biological Supply) plus a piece of *Agaricus bisporus* (Lange) mushroom, which has no detectable antibiotic activity. After the F1 adults from a given replicate vial had aged for 10 days, one F1 female and one *ST-1* male were placed in each of three vials of standard medium. Thus, for each mushroom species tested, a total of nine vials were set up with F1 females, three from each of the three replicate vials (Fig. 1). All F2 progeny were sexed and counted. The occurrence of males in the F2 suggests that their F1 mother may have been cured as a result of developing in a particular mushroom species.

Because maternal transmission of *Wolbachia* is slightly less than 100% (Dyer and Jaenike, 2004), a small fraction of the F1 females were expected to produce sons as well as daughters. To distinguish antibiotic curing from stochastic incomplete transmission of the *Wolbachia* infection, the F2 sex ratios produced by different F1 females were compared. If only a single F1 female (e.g. replicate 1b in Fig. 1) produced F2 sons, then it is most likely that the infection was not transmitted to that F1 female. If all of the F1 females from one of the first-generation replicates (e.g. replicates 1a, 1b and 1c in Fig. 1) produced sons, but none of the females from the other replicates did, this would suggest that the original *mk-3* female used to set up that replicate was herself uninfected. Finally, if sons were produced by one or

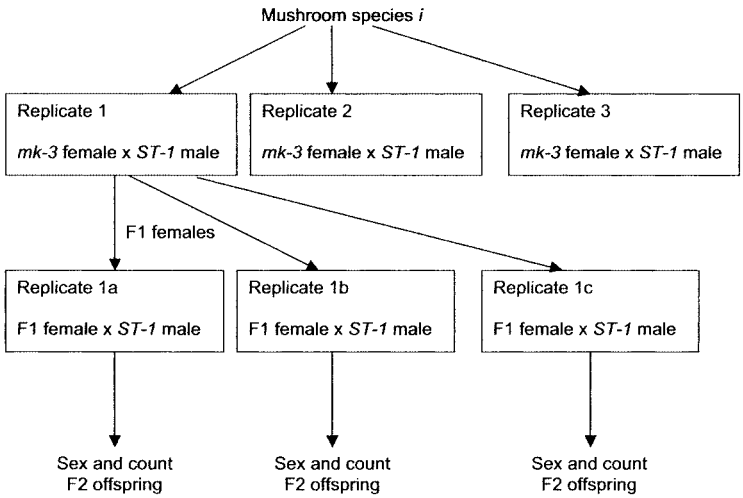

Fig. 1. Experimental design to detect the presence of *Wolbachia*-curing antibiotics in mushrooms. For each mushroom species, replicates 2 and 3 were followed as for replicate 1.

more F1 females from the different generation 1 replicates, this would suggest that the mushroom on which they developed cured them of their *Wolbachia* infections.

One mushroom species in our 2004 sample, *Gyrodon merulioides* (Schweinitz) Singer, met this last criterion. To determine whether the production of F2 males was due to chance or was due to antibiotics in *G. merulioides*, we collected this species again in 2005 from three different sites in the Rochester area and tested for antibiotic activity again with *Wolbachia*-infected *mk-3* females.

The use of mushrooms collected in New York, instead of Arizona where *D. innubila* occurs, was done for logistical reasons. Moreover, using mushrooms from outside the range of *D. innubila* ensures that all emergent *D. innubila* are offspring of the *Wolbachia*-infected females used in our experiments, and not the offspring of wild females whose infection status could not be known. Finally, there is considerable similarity between the mushrooms of Arizona and New York: among the 84 mushrooms identified to species in our samples, at least 45 also occur in Arizona (Gilbertson and Bigelow, 1998; Bates, 2006). Thus, the chemical diversity of our samples is probably similar to a comparable sample size of mushrooms from Arizona.

RESULTS

Drosophila innubila were able to complete development on a wide variety of mushrooms, indicating that they can survive exposure to different chemical environments during larval development. Among the F2 flies – those in which the effects of antibiotic curing of *Wolbachia* would be evident – there were 6976 females and 283 males in the initial screens (Table 1). The overall proportion of males (3.9%) is close to the proportion of male offspring (3.1%) produced by wild-caught infected females (Dyer and Jaenike, 2004). This indicates that the vast majority of mushrooms had insufficient antibiotic activity to cure *D. innubila* of their *Wolbachia* infection.

A substantial number of the F2 males (178) were produced by three females that had developed on just two of the mushroom species we assayed – *Lactarius vinaceorufescens* Smith and *Gyrodon merulioides* – thus making these mushrooms of particular interest. For *L. vinaceorufescens*, the F2 female to male offspring numbers produced by the three F1 females that did produce offspring were 374 : 0, 122 : 108, and 66 : 1. Because two of the three F1 females produced essentially all-female progeny, it is likely that the female with a normal offspring sex ratio did not inherit her mother's *Wolbachia* infection, not because of having developed on *L. vinaceorufescens*.

In 2004, two of the unrelated females (i.e. from separate generation 1 replicates) that had developed on *Gyrodon merulioides* produced 11 : 10 and 57 : 60 female to male offspring sex ratios, although the remaining four F1 females produced strongly female-biased sex ratios. The production of males by two females suggests that *G. merulioides* may contain antibiotics that cure flies of *Wolbachia*. Therefore, we collected and assayed *G. merulioides* again in 2005. We tested 78 F1 females from 26 independent replicates. The F2 progeny from these females included 2391 females and 7 males. Thus, the 2005 samples of *G. merulioides* did not contain sufficient levels of antibiotics to cure *D. innubila* of their *Wolbachia* infections.

DISCUSSION

The vast majority of the ~100 species of mushrooms tested provided no evidence of curing *D. innubila* of their *Wolbachia* infections, as very few or no male offspring were produced

Table 1. The numbers of F2 male and female offspring resulting from the development of *Wolbachia*-infected *Drosophila innubila* females on different species of mushrooms

		F2 offspring	
Mushroom species	Year	Females	Males
Agaricus arvensis	2004	102	3
Amanita bisporigera	2003	1004	0
Amanita brunnescens	2003	40	0
Amanita brunnescens	2004	9	0
Amanita citrina	2003	748	15
Amanita citrina	2004	18	0
Amanita flavoconia	2004	138	1
Amanita gemmata	2003	742	0
Amanita muscaria	2003	1463	17
Amanita muscaria	2004	243	1
Amanita phalloides	2003	149	0
Amanita phalloides	2004	99	3
Amanita virosa	2004	42	0
Armillaria caligata	2004	157	0
Armillaria sp.	2003	656	0
Armillaria sp.	2003	311	0
Armillaria sp.	2004	156	0
Boletus chrysenteron	2003	233	0
Boletus chrysenteron	2004	57	2
Boletus edulis	2004	1	1
Boletus griseus	2003	638	14
Boletus innixus	2004	86	3
Boletus innixus	2004	143	2
Boletus ornatipes	2003	667	0
Cantharellus cibarius	2003	165	0
Cantharellus cibarius	2004	108	4
Clavicorona pyxidata	2003	79	0
Clitocybe nuda	2004	70	2
Clitopilus prunulus	2003	25	0
Clavulina sp.	2003	71	4
Coprinus micaceus	2004	103	4
Cortinarius alboviolaceus	2003	131	0
Cortinarius pseudosalor	2004	4	1
Cortinarius sp.	2003	172	0
Cryptoporus volvatus	2004	75	2
Daedaleopsis confragosa	2004	99	0
Dictyophora duplicata	2003	33	0
Entoloma abortivum	2003	28	0
Fuscoboletinus aeruginascens	2003	138	0
Grifola frondosa	2003	919	0
Grifola frondosa	2004	25	1
Gymnopilus spectabilis	2003	33	0
Gyrodon merulioides	2004	186	72

Table 1.—*continued*

Mushroom species	Year	F2 offspring	
		Females	Males
Gyrodon merulioides	2005	2391	7
Gyroporus castaneus	2004	27	1
Helvella infula	2003	115	11
Hericium coralloides	2003	8	0
Hericium sp.	2004	1	0
Hygrocybe conica	2004	82	0
Hygrocybe flavescens	2003	46	0
Hygrocybe sp.	2004	219	2
Hygrophorus ebureneus	2004	132	3
Hygrophorus perplexus	2003	612	0
Hygrophorus russula	2003	1182	0
Hypholoma sublateritium	2003	30	0
Hypomyces chrysospermus	2004	93	1
Hypomyces hyalinus	2004	49	0
Hypsizygus tessulatus	2004	1	1
Lactarius chrysorheus	2003	1017	0
Lactarius chrysorheus	2003	201	0
Lactarius deceptivus	2004	2	1
Lactarius deliciosus	2003	261	0
Lactarius hygrophoroides	2004	13	1
Lactarius indigo	2004	80	2
Lactarius rufus	2003	250	0
Lactarius vinaceorufescens	2003	496	108
Lactarius vinaceorufescens	2004	66	1
Lactarius sp.	2003	213	0
Laetiporus sulphureus	2003	51	0
Lentinellus ursinus	2004	70	1
Lepiota rachodes	2004	89	1
Lepiota sp.	2003	4	0
Lyophyllum decastes	2003	266	0
Meripilus giganteus	2004	34	2
Mycena leaiana	2004	103	22
Mycena sp.	2003	71	0
Mycena sp.	2003	153	0
Naematoloma capnoides	2003	17	0
Naematoloma sublateritium	2004	112	3
Omphalotus olearius	2003	879	0
Omphalotus olearius	2004	58	1
Paxillus involutus	2004	74	4
Paxillus involutus	2004	180	3
Pholiota squarrosa	2004	67	0
Pholiota squarrosoides	2003	606	0
Pholiota sp.	2003	22	5
Pleurocybella porrigens	2004	20	0
Polyporus squamosus	2004	2	1

Table 1.—*continued*

Mushroom species	Year	F2 offspring	
		Females	Males
Psathyrella conissans	2003	148	0
Rozites caperata	2003	228	0
Rozites caperata	2004	125	1
Russula brevipes	2004	132	1
Russula mariae	2004	237	2
Russula silvicola	2003	1330	3
Russula sp.	2003	729	0
Scleroderma citrinum	2004	36	0
Suillus americanus	2004	77	2
Suillus granulatus	2004	133	3
Suillus grevillei	2003	633	0
Suillus grevillei	2004	64	0
Suillus intermedius	2004	153	2
Suillus pictus	2004	202	4
Suillus punctipes	2004	32	2
Tremellodendron pallidum	2004	50	0
Tricholoma caligatum	2003	315	0
Tricholoma flavovirens	2003	134	0
Tricholomopsis sulphureoides	2003	62	0
Tubaria furfuracea	2004	36	0
Tylopilus felleus	2004	111	2
Tylopilus sp.	2003	348	0
Tyromyces chioneus	2004	2	1
Xerula furfuracea	2003	240	0
Xerula megalospora	2004	31	2

Note: For each year in which a given species of mushroom was tested, the data for all F2 families have been pooled.

in the F2 generation. For only two species – *Lactarius vinaceorufescens* and *Gyrodon merulioides* – were significant numbers of males produced in the F2 generation.

For *L. vinaceorufescens*, only one of three independent females tested produced a substantial number of sons. Because *Wolbachia* is transmitted with <100% fidelity, it is likely that the one female that produced sons happened by chance not to have inherited the infection. In our initial 2004 test, two of six females that had developed on *Gyrodon merulioides* produced normal sex ratios. In our follow-up assay in 2005, all of the 26 females that developed on this species produced few or no sons. Thus, it is likely that the production of sons by two females in 2004 was the result of chance failure to inherit the *Wolbachia* infection, rather than antibiotic curing by having developed on *G. merulioides*. Across all mushroom species, only three F1 females out of 270 tested produced normal sex ratios, and this is actually slightly fewer than expected on the basis of our 3% empirical estimation of stochastic incomplete maternal transmission (Dyer and Jaenike, 2004).

Our failure to find any mushroom species with significant antibiotic activity against *Wolbachia* contrasts with other surveys of mushrooms that found numerous species with

antimicrobial activity (Suay *et al.*, 2000; Rosa *et al.*, 2003). The negative results in our survey are not due to the *Wolbachia* in *D. innubila* being resistant to antibiotics, as the development of female larvae on medium containing as little as 0.5 μmol$\cdot$l^{-1} of either tetracycline or rifampicin results in the production of substantial numbers of male offspring (Dyer *et al.*, 2005). Thus, these *Wolbachia* are highly sensitive to certain antibiotics.

We suggest that the endosymbiotic habitat of *Wolbachia* confers protection from many antimicrobial compounds, as such compounds must pass intact from the host insect's digestive system to the hemocoel and from there to the cells of the germline, where the *Wolbachia* are resident. It is likely that some potential antibiotics are degraded enzymatically in the gut or are sensitive to gut pH, experience poor absorption from the gut due to size or solubility, or have poor penetration of the germline cells in which *Wolbachia* are resident, as comparable effects are known in mammals (Zhang and Benet, 2001). It is thus interesting to note that tetracycline, the ingestion of which can cure *D. innubila* of their *Wolbachia* infections, can survive passage and be taken up through the vertebrate gut (Pindell *et al.*, 1959).

That none of the mushrooms we tested cure *D. innubila* of *Wolbachia* is relevant to understanding the evolution and ecology of these infections in natural populations. First, if some mushrooms did cure flies of their *Wolbachia* infections, there would be strong selection on females to oviposit on such mushrooms, as this would rescue nuclear genes from the genetic black hole associated with male killing (Sullivan and Jaenike, 2006). However, because mushrooms have little or no antibiotic activity against *Wolbachia*, the evolution of oviposition site preference is likely to be governed by factors other than potential antibiotics. This is in contrast to the selective advantage of utilizing mushrooms that contain toxins (e.g. α-amanitin) that kill *Drosophila*-parasitic nematodes (Jaenike, 1985).

A second consequence of our findings is that the fidelity of *Wolbachia* transmission at the population level will not depend on the particular species of available mushrooms, which can vary greatly among habitats, between seasons, and from one year to the next (Orlos, 1975). Therefore, because transmission fidelity is an important determinant of infection dynamics (Dyer and Jaenike, 2004), the prevalence of infection in natural populations probably varies much less than if *Wolbachia* transmission were a function of mushroom species. Consequently, to understand the temporal and spatial variation in *Wolbachia* prevalence in natural populations of *D. innubila* (Dyer and Jaenike, 2005), we must look elsewhere.

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REFERENCES

Bates, S.T. 2006. A preliminary checklist of Arizona macrofungi. *Canoita*, **2**: 47–69.

Clancy, D.J. and Hoffmann, A.A. 1998. Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. *Entomol. Exp. Applic.*, **86**: 13–24.

Dyer, K.A. and Jaenike, J. 2004. Evolutionarily stable infection by a male-killing endosymbiont in *Drosophila innubila*: molecular evidence from the host and parasite genomes. *Genetics*, **168**: 1443–1455.

Dyer, K.A. and Jaenike, J. 2005. Evolutionary dynamics of a spatially structured host–parasite association: *Drosophila innubila* and male-killing *Wolbachia*. *Evolution*, **59**: 1518–1528.

Dyer, K.A., Minhas, M. and Jaenike, J. 2005. Expression and modulation of embryonic male-killing in *Drosophila innubila*: opportunities for multi-level selection. *Evolution*, **59**: 838–848.

Gilbertson, R.L. and Bigelow, D.M. 1998. Annotated checklist of wood-rotting basidiomycetes of the sky islands of southeastern Arizona. *J. Arizona-Nevada Acad. Sci.*, **31**: 13–36.

Hurst, L.D. 1991. The incidences and evolution of cytoplasmic male-killers. *Proc. R. Soc. Lond. B*, **244**: 91–99.

Hurst, G.D.D. and Jiggins, F.M. 2000. Male-killing bacteria in insects: mechanisms, incidence and implications. *Emerg. Infect. Dis.*, **6**: 329–336.

Jaenike, J. 1985. Parasite pressure and the evolution of amanitin tolerance in *Drosophila*. *Evolution*, **39**: 1295–1301.

Orlos, H. 1975. *Forest Fungi Against the Background of Environment*. Warsaw, Poland: Foreign Scientific Publications Department of the National Center for Scientific, Technical and Economic Information.

Pindell, M.H., Cull, K.M., Doran, K.M. and Dickison, H.L. 1959. Absorption and excretion studies on tetracycline. *J. Pharmacol. Exp. Therapeutics*, **125**: 287–294.

Rosa L.H., Machado, K.M.G., Jacob, C.C., Capelari, M., Rosa, C.A. and Zani, C.L. 2003. Screening of Brazilian basidiomycetes for antimicrobial activity. *Memorias do Instituto Oswaldo Cruz*, **98**: 967–974.

Suay, I., Arenal, F., Asensio, F.J., Basilio, A., Cabello, M.C., Diez, M.T. *et al.* 2000. Screening of basidiomycetes for antimicrobial activities. *Antonie van Leeuwenkoek Int. J. General Molec. Microbiol.*, **78**: 129–139.

Sullivan, J. and Jaenike, J. 2006. Male-killing *Wolbachia* and male mate choice: a test with *Drosophila innubila*. *Evol. Ecol. Res.*, **8**: 91–102.

Turelli, M. 1994. Evolution of incompatibility-inducing microbes and their hosts. *Evolution*, **48**: 1500–1513.

Werren, J.H. 1997. Biology of *Wolbachia*. *Annu. Rev. Entomol.*, **42**: 537–609.

Zhang, Y. and Benet, L.Z. 2001. The gut as a barrier to drug absorption: combined role of Cytochrome P450 3A and P-Glycoprotein. *Clin. Pharmacokinetics*, **40**: 159–168.