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Several bicyclic compounds with structures similar to the characteristic products were also present in the pyrolysates, including 1,2,3,4-tetrahydro-1,5,6-trimethyl-naphthalene (Fig. 1, IX), trimethyl-naphthalenes (Fig. 1, X), 1,2,3,4-tetrahydro-1,1,5,6-tetramethyl-naphthalene (Fig. 1, XI), and 1,2,3,4-tetrahydro-1,1,6-trimethyl-naphthalene (Fig. 1, XII), among others. These compounds are interpreted to be the pyrolysis products of degraded labdanoids within the macromolecular structure and generally differ by having fewer methyl groups and/or by having increased aromaticity. The presence of these compounds is not surprising, considering the age and maturity of these samples.

In addition to the characteristic labdanoid products, several abietane-class diterpenes were also identified in the pyrolysates. These included dehydroabietane (Fig. 1, XIII), methyl dehydroabietate (Fig. 1, XIV), methyl callitrate (Fig. 1, XV), related compounds (Fig. 1, XVI and XVII α and β), and others. These compounds are common in Class I ambers (1, 3), as well as in modern resins (12). The distributions of these products in pyrolysates generated by pyrolysis at temperatures $T_{py} = 300^{\circ}\text{C}$ and $T_{py} = 480^{\circ}\text{C}$ are essentially identical, indicating that these diterpenes are present in these samples as occluded materials. The exact stereochemistry of dehydroabietane (Fig. 1, XIII) or methyl 16,17-dinor dehydroabietate and methyl 16,17-dinor callitrate (Fig. 1, XVII α and β) is unknown in these samples, but is drawn in Fig. 1 as commonly found in Class I ambers.

The observation of Class Ic ambers in Carboniferous sediments suggests that preconifer gymnosperms were using complex polyterpenoid resin in a manner similar to that seen in a wide variety of modern species. Modern resins that are structurally analogous to Class Ic ambers are primarily derived from angiosperms. Our data do not imply that angiosperms existed in the Carboniferous, because the fossil record does not record unequivocal angiosperm fossils until the Cretaceous. However, our data do suggest that the divergence of the biosynthetic mechanisms needed to produce resins based on regular and enantio-series labdanoid diterpenes predates both the emergence of true conifers and the differentiation of angiosperms and gymnosperms. Furthermore, these basic biosynthetic pathways have been retained in both gymnosperms and angiosperms through several major extinction events and over 300 million years of evolution. Based on genomic evidence, previous workers have postulated initial differentiation of terpene synthase genes associated with the production of resin-related diterpenes during the Carboniferous (14). Our data support this hypothesis.

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11. Despite the common terminology used to describe labdanoids related to communic acids as regular and those related to ozic acid as entatio, these products are in fact epimeric, not enantiomers. The same is true of the bicyclic products (Fig. 1, I to IV and V to VIII) derived from the polymers of these terpenoids by Py-GC-MS. This terminology, although imprecise, is deeply entrenched in the literature related to these compounds and is retained here for consistency with numerous previously published works.
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15. This contribution constitutes part 14 of the authors' series of publications under the general title, "The Nature and Fate of Natural Resins in the Geosphere."

Supporting Online Material

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Immune Activation by Life-Shortening *Wolbachia* and Reduced Filarial Competence in Mosquitoes

Zakaria Kambris, Peter E. Cook, Hoang K. Phuc, Steven P. Sinkins*

Wolbachia strain wMelPop reduces the longevity of its *Drosophila melanogaster* host and, when introduced into the mosquito *Aedes aegypti*, halves its life span. We show that wMelPop induces up-regulation of the mosquito's innate immune system and that its presence inhibits the development of filarial nematodes in the mosquito. These data suggest that wMelPop could be used in the global effort to eliminate lymphatic filariasis and possibly for the control of other mosquito-borne parasites where immune preactivation inhibits their development. The cost of constitutive immune up-regulation may contribute to the life-shortening phenotype.

Wolbachia pipientis is a maternally inherited intracellular bacterium of invertebrates, capable of spreading itself through populations by reproductive manipulation such as cytoplasmic incompatibility (CI). The strain wMelPop or "popcorn," unusually, reduces the longevity of its *Drosophila melanogaster* host (1) and also has been shown to halve life-span when the mosquito *Aedes aegypti* was stably trans-

infected (2). The wMelPop life-shortening phenotype offers the prospect of a disease control system by potentially skewing the population structure toward younger individuals. Vectorial capacity is particularly sensitive to mosquito age because mosquito-borne pathogens require an extrinsic incubation period between ingestion and transmission that is long relative to mean life-span in the field, such that only older mosquitoes within

a population are potentially infective. wMelPop was also found to be inherited at high rates and to induce strong CI in *Ae. aegypti*, which provides a reproductive advantage to infected females. The wMelPop strain should be capable of spreading through populations despite the reduction in mean life-span, because reproduction by older individuals makes a relatively small contribution to the next generation (2–6).

We compared host gene expression using whole-genome microarrays in genetically identical *Ae. aegypti* lines infected and uninfected with wMelPop (7) to examine the mechanism underlying the life-shortening phenotype. Of 199 gene transcripts up-regulated by more than a twofold threshold, 78 had putative immune-related functions (Fig. 1 and table S2). These included genes that encode 17 CLIP domain, serine proteases, nine FREPs (fibrinogen-related proteins), six cecropins, four TEPs (thioester-containing proteins), three defensins, three PPOs (prophenoloxidases), two lysozymes, two PGRPs (peptidoglycan recog-

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nitiation proteins), two GNBP (Gram-negative binding proteins), and the nuclear transcription factor *Rel2*. Various effector genes, particularly cecropins and other antimicrobial peptides, show the highest up-regulation (Fig. 1 and table S2). Five immune-related genes (primarily of predicted regulatory function) were down-regulated below the twofold threshold (table S2).

Quantitative RT-PCR (qRT-PCR) experiments with mosquitoes at 2 and 15 days post eclosion using a subset of immune-related genes, provided broad support for the array data (Fig. 2 and table S1). Up-regulation occurred at similar levels at both age points. This scale of constitutive or chronic immune up-regulation is unexpected (the short-lived acute immune gene expression is commonly observed after septic challenge). In *Drosophila simulans* and *Aedes albopictus*, naturally occurring *Wolbachia* was found neither constitutively to induce nor to suppress the transcription of various inducible antibacterial genes (8). Constitutive up-regulation of immune genes is known to have a high cost, and trade-offs between longevity and pathogen resistance associated with immune up-regulation have been described in *D. melanogaster* (9). Therefore, constitutive up-regulation of immune genes associated with *wMelPop* infection also provides a

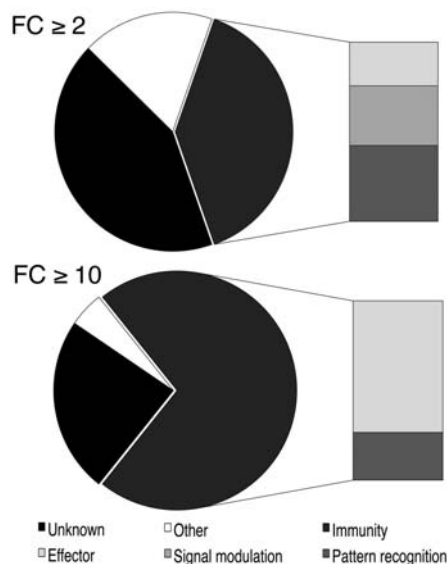


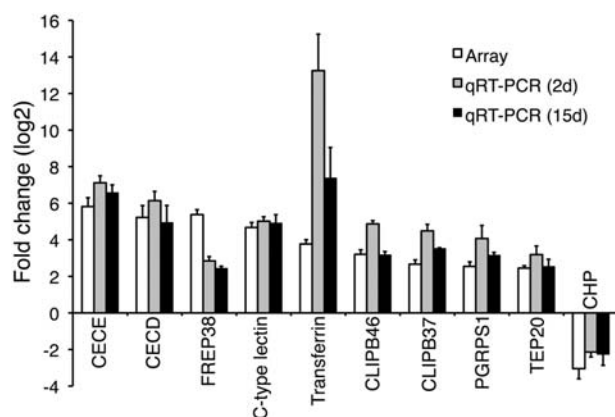
Fig. 1. Induction of immune gene transcription in *wMelPop*-infected female *Ae. aegypti*. A summary of data from four replicate *Ae. aegypti* microarrays is shown as gene transcripts significantly up-regulated in *wMelPop*-infected versus uninfected female mosquitoes. Of 199 up-regulated gene transcripts with fold change (FC) ≥ 2 ($P < 0.05$), a considerable proportion had a putative immune function (immunity) compared with genes of other known function (other) and unknown function (unknown). Gene transcripts with immune-related function were categorized into effectors, signal modulation, and pattern recognition. Gene transcripts encoding immune effectors dominated the list of highly up-regulated genes (FC ≥ 10).

possible mechanism for, or contributory factor to, the life-shortening phenotype.

The development of various pathogens is known to be inhibited by immune preactivation in mosquitoes, which suggests that the constitutive immune regulation observed could influence the transmission of these pathogens to humans. For example, when *Ae. aegypti* was injected with bacteria 24 hours before challenge with *Brugia* filarial nematodes, causative agents of human lymphatic filariasis, prevalence and mean intensity of infection were reduced significantly (10). Synthetic cecropin peptides also have known filarial-killing activity (11); data from the microarray experiment showed that six cecropin genes were all up-regulated by more than 25-fold in the presence of *wMelPop* (table S2).

We investigated the effect of *wMelPop* on filarial transmission. A filarial susceptibility locus from the susceptible Ref^m strain of *Ae. aegypti* was backcrossed into the *wMelPop*-infected and control uninfected backgrounds by using a visible marker linked to the susceptibility locus (7). A blood meal infected with *Brugia pahangi* microfilariae (a rodent filarial nematode previously shown to be a good model for studies of the genetics of susceptibility to infection by human filariae) was provided (12). In comparison with the uninfected control line, significant reductions in the mean numbers of third larval stage (L3) infective worms, as well as in the prevalence of infected mosquitoes, were observed at three microfilarial densities when the *wMelPop* infection was present (Fig. 3). The inhibition effect was strongest at the lowest microfilarial density used, 11 microfilariae per microliter of blood, with 79.8% (A bars) or 84.2% (B bars) reductions in mean L3 stage larvae in the *wMelPop*-infected line compared with the *wMelPop*-uninfected control. All microfilarial densities used are high compared with blood densities that would occur naturally.

Fig. 2. Microarray and qRT-PCR analyses of differentially regulated genes. The differential regulation of transcript levels in *wMelPop*-infected *Ae. aegypti* versus a genetically identical uninfected *Ae. aegypti* line was examined for a selection of nine up-regulated genes corresponding to two cecropins (CECE, AAEL000611; CECD, AAEL000598), a fibrinogen- or fibronectin-related protein (FREP38, AAEL0015428), a C-type galactose-specific lectin (AAEL005641), a transferrin (AAEL0015458), two CLIP-domain serine proteases (CLIPB46 AAEL005431, CLIPB37 AAEL005093), a peptidoglycan recognition protein (PGRPS1, AAEL009474), a thioester-containing protein (TEP20, AAEL001794), and a conserved hypothetical protein that was down-regulated (CHP, AAEL003467). The values shown are the means (\pm SEM) of three different qRT-PCR experiments with independent samples, at 2 and 15 days post eclosion.



Filarial nematodes are somewhat harmful to mosquitoes. To investigate whether the presence of *wMelPop* conferred protective effects against other insect pathogens, the two lines were challenged by thoracic pricking with a virulent strain of the Gram-negative bacterium *Erwinia carotovora*. There was higher survival when *wMelPop* was present than in the *wMelPop*-free control (Fig. 4). A control challenge with the Gram-positive bacterium *Micrococcus luteus* confirmed that the high mortality seen when the *wMelPop*-uninfected line was challenged with *Erwinia* was not simply a result of septic injury.

Any protective effects provided against harmful pathogens commonly encountered by mosquitoes will act to increase the population-spreading capacity of the *wMelPop* strain. However, any benefits accrued from pathogen protection in nature are likely only partially to offset the fitness costs associated with immune gene expression. The immune activation phenotype may be a side effect of the unusually fast replication of *wMelPop* and/or because the immune evasion strategies normally used by *Wolbachia* are impaired in this novel host. *wMelPop Wolbachia* must itself be at least partly resistant to the immune effectors induced, because it is maternally transmitted at high levels in infected *Ae. aegypti* (2).

Wolbachia infections, including *wMelPop*, were recently reported to provide a protective function against pathogenic viruses in *Drosophila* (13, 14). The up-regulation of immune genes provides a possible explanation or contributory factor, because there is a degree of overlap between immune peptides induced by bacteria and viruses (15, 16). Cecropins have previously been shown to have antiviral effects, for example, inhibiting HIV-1 (human immunodeficiency virus 1) replication (17), and an artificial cecropin-defensin hybrid peptide inhibited dengue virus replication (18). Knockdown studies will allow

these hypotheses to be tested. Cecropins are also known to have a major inhibitory effect on *Plasmodium* development (19, 20). Furthermore, the orthologs of *Rel2*, *TEP20* (gene identifier AAEL001794) (7), and *LRIM1* (AAEL012086), all up-regulated in the presence of *wMelPop*, have been shown by knockdown studies in *Anopheles* to regulate the intensity of *Plasmodium* infection (21–26), as has mosquito midgut microbiota by means of immune stimulation (27). *wMelPop*-transinfected *Anopheles* species should be tested for their ability to inhibit transmission of *Plasmodium* in order to evaluate this malaria control strategy.

Around 120 million people across the tropics are infected with the filarial nematode species that cause lymphatic filariasis, a leading global cause of disability. *Ae. aegypti* is not a natural vector of the disease; however, other *Aedes* species are primary regional vectors, such as *Ae. polynesiensis* in the South Pacific region. The need for novel control methods for *Ae. polynesiensis* has been previously highlighted (28). Sustained efforts to eliminate filariasis in the region by mass drug administration alone have failed; this

is thought to be because *Ae. polynesiensis* is such an efficient vector of *Wuchereria bancrofti*, the main causative agent of human lymphatic filariasis, at low microfilarial densities. Another attractive target is *Culex quinquefasciatus*, the major urban vector of *W. bancrofti* across the tropics. Once stable *wMelPop* infections are created, they would need to be challenged directly with *W. bancrofti*, because it remains possible that the effects observed are species-specific, but the prospect of a self-spreading transmission-reduction system is attractive, particularly given that it is likely to be especially effective when microfilarial densities in the human population are low.

The data reported here suggest *wMelPop* may represent a valuable tool in the global effort to eradicate lymphatic filariasis and possibly for the control of other mosquito-borne parasites. The combination of direct inhibitory effects on filariae in the mosquito, together with life-span shortening, could have a powerful overall impact in reducing disease transmission. The ability of *wMelPop*, in common with many other *Wolbachia* strains, to drive itself through

populations using CI makes this a very attractive strategy.

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Fig. 3. *wMelPop*-infected mosquitoes show a reduction in filarial infection prevalence and intensity. The mean numbers (\pm SEM) of infective third (L3) stage *Brugia pahangi* larvae are shown at 10 to 13 days post microfilarial challenge in *Ae. aegypti* *wMelPop*-infected (Ae_Pop) versus uninfected (Ae_Tet) lines, that are filaria-susceptible after backcrossing with the Ref^m strain. Four independent challenge experiments are shown with microfilarial densities (microfilariae per microliter blood) of 11 (experiments A and B), 13 (C), and 23 (D). Values above bars show the prevalence of filarial infection as a proportion of mosquitoes that contained at least one L3 *Brugia* larva versus the total number of mosquitoes dissected in each category. Differences were significant at * $P < 0.01$ or ** $P < 0.001$ (Mann-Whitney *U* test).

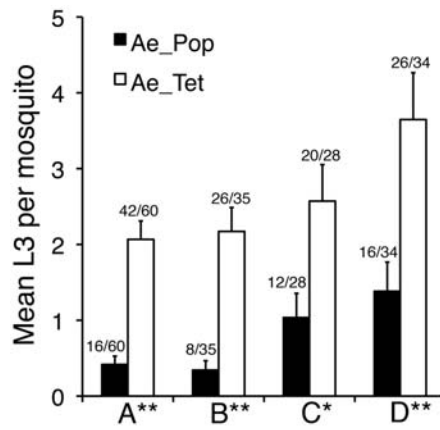
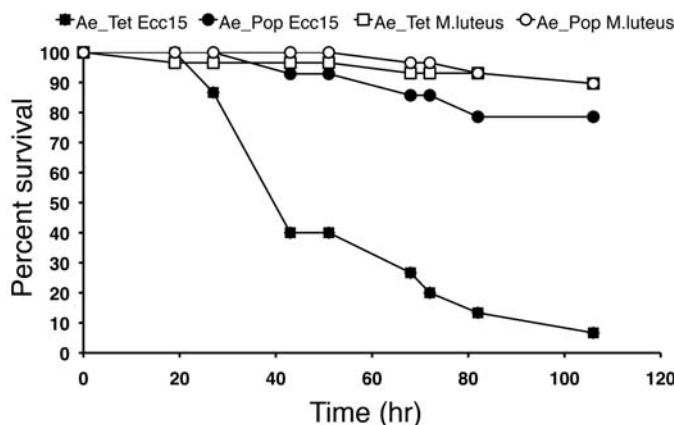


Fig. 4. Increased resistance to a pathogenic bacterium in *wMelPop*-infected mosquitoes. The daily survival rates (%) of *Ae. aegypti* carrying *wMelPop* (Ae_Pop) were compared with the *Wolbachia*-free line (Ae_Tet) after infection with *E. carotovora 15* (Ecc15), a pathogenic Gram-negative bacterium. The horizontal axis represents the incubation time after infection in hours. Control infections with the Gram-positive *M. luteus* demonstrate that the early death or protection effects are not due to septic wounding alone.



Supporting Online Material

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 References

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