Pervasive effects of *Wolbachia* on host activity

Michael T.J. Hague^{1,*}, H. Arthur Woods¹, and Brandon S. Cooper¹

¹Division of Biological Sciences
University of Montana
32 Campus Dr.
Missoula, MT 59812

***Corresponding Author**

Michael T.J. Hague
Division of Biological Sciences, University of Montana
32 Campus Dr. HS 104, Missoula, MT 59812
(406) 243-5122
michael.hague@mso.umt.edu

ABSTRACT

Heritable symbionts have diverse effects on the physiology, reproduction, and fitness of their hosts. Maternally transmitted *Wolbachia* are one of the most common endosymbionts in nature, infecting about half of all insect species. We test the hypothesis that *Wolbachia* alter host behavior by assessing the effects of 14 different *Wolbachia* strains on the locomotor activity of nine *Drosophila* host species. We find that *Wolbachia* alter the activity of six different host genotypes, including all hosts in our assay infected with *w*Ri-like *Wolbachia* strains (*w*Ri, *w*Suz, *w*Aur), which have rapidly spread among *Drosophila* species in only the last 13,000 years. While *Wolbachia* effects on host activity were common, the direction of these effects varied unpredictability and sometimes depended on host sex. We hypothesize that the prominent effects of *w*Ri-like *Wolbachia* may be explained by patterns of *Wolbachia* titer and localization within host somatic tissues, particularly in the central nervous system. Our findings support the view that *Wolbachia* have wide-ranging effects on host behavior. The fitness consequences of these behavioral modifications are important for understanding the evolution of host-symbiont interactions, including how *Wolbachia* spread within host populations.

INTRODUCTION

Insects harbor microorganisms that have wide-ranging effects on their performance and fitness [1–3], including manipulations to reproduction [4–7], provisioning of nutrients [1,8,9], modifications of thermotolerance [10,11], and defense against pathogens [12–15]. Microbes may also alter host behavior [16–21]. In extreme instances, parasitic microbes can induce behaviors that increase the likelihood of transmission—for example, by directing hosts to habitats that promote transmission [22–28]. Infected hosts may also change their own behavior as an immune strategy against infection, including seeking warm temperatures to induce a “behavioral fever” [29,30] or reducing activity and increasing sleep time [19,31–34]. Such behavioral modifications have important implications for microbe spread and host fitness.

Maternally transmitted *Wolbachia* are the most common endosymbionts in nature, infecting many arthropods [5,35,36] and two distantly related groups of nematodes [37]. Discordant *Wolbachia* and host phylogenies indicate that many hosts have recently acquired *Wolbachia* via introgressive and horizontal transfer [38–43]. *Wolbachia* are primarily transmitted vertically by female hosts, so natural selection favors beneficial effects on host fitness that promote spread [44–47]. Maternal transmission occurs via the host reproductive system, but *Wolbachia* are also found in host somatic tissues, including nervous, digestive, and metabolic tissues [48–51]. Still, the behavioral and physiological consequences of somatic infections are poorly understood [19,51].

Prior work indicates *Wolbachia* influence several host behaviors [19,52,53], including sleep [54–56] and temperature preference [20,57,58]. We broadly test for *Wolbachia* effects on the locomotor activity of *Drosophila* hosts infected with A-group *Wolbachia* ($N = 11$), B-group *Wolbachia* ($N = 1$), and an A- and B-group co-infection ($N = 1$). Our analysis includes two prominent A-group clades that recently spread among *Drosophila*: wMel-like *Wolbachia* (wMelCS, two wMel variants, wYak, wSan, and wTei) and wRi-like *Wolbachia* (wRi, wSuz, and wAur) [42,43]. We find that *Wolbachia* effects on host activity are common, particularly for wRi-like *Wolbachia*, a “super-spreader” strain that rapidly spread among *Drosophila* species in the last ~13,000 years [42].

METHODS

Fly lines

We evaluated 13 different *Wolbachia*-infected host genotypes (Figure 1, Table S1), consisting of nine *Drosophila* species infected with 14 different A- and B-group *Wolbachia* that diverged up to 46 million years ago [59]. For two of the host species, *D. melanogaster* and *D. simulans*, we tested multiple *Wolbachia*-infected genotypes. This included a *D. simulans* host co-infected with A-group wHa and B-group wNo [60–63]. We used tetracycline treatment as previously described [20] to generate uninfected genotypes to pair with each infected genotype, while taking care to avoid detrimental effects of the antibiotic treatment on mitochondrial function [64] (see Supplemental Methods).

Host locomotor activity assays

We reared flies at 25°C under a 12-h light:12-h dark cycle (Percival model I-36LL) on a standard food diet [20]. Each day, we collected a batch of female and male virgins for one pair of uninfected and infected genotypes. The four treatment groups (uninfected females, infected females, uninfected males, and infected males) were maintained in isolation until they were 3 to 5 days old. We then measured the locomotor activity of the batch of flies using a 16-chamber flow-through respirometry and data acquisition system (MAVEN, Sable Systems International). The MAVEN has 16 2.4 ml volume polycarbonate animal chambers and an activity board that uses infrared light (invisible to flies) to monitor animal activity in each chamber, sampled at 1 Hz (Figure S1). Individual flies were aspirated into a randomly assigned chamber and allowed to adjust to the new environment for 0.5 hours. Activity measurements were then recorded over a 3-hour period between the hours of 0900 and 1600.

The raw outputs from the activity sensors were transformed into the activity index absolute distance sums (ADS). We calculated ADS by first calculating the cumulative sum of the absolute difference between consecutive activity readings, and then calculating the slope of cumulative activity vs. time [65,66]. We used mean ADS over the 3-hour period as our estimate of locomotor activity for each fly; however, our analyses were robust regardless of how we quantified activity (see Supplemental Methods). We found that the mean ADS activity data required a transformation for statistical analysis; however, a single data transformation was not suitable for all host species. We used a log transformation of mean ADS for *D. simulans*, *D.*

suzukii, *D. auraria*, *D. mauritiana*, and *D. sechellia*, and a square root transformation for *D. melanogaster*, *D. yakuba*, *D. santomea*, and *D. teissieri*. We present a full statistical analysis of all datasets in Tables S2 and S3, respectively.

We used the log- and square root-transformed mean ADS data as dependent variables in linear models. We included infection status, sex, and an infection-by-sex interaction effect as independent variables, as well as additional independent variables to account for other potential sources of activity variation: randomly assigned animal chamber (1-16), experimental start time, mean water vapor (ppt), mean relative humidity (%), mean temperature (°C), and mean light intensity (lux) [65,66]. We evaluated the significance of individual effects using *F* tests and type III sum of squares using the “Anova” function in the *car* R package [67,68].

Wolbachia phylogenomic analysis

We used publicly available *Wolbachia* genome assemblies [20,42,43,59,69–71], and new Illumina sequencing, to generate a Bayesian phylogram [20] (see Supplemental Methods). *Wolbachia* effects on host activity were especially common for *w*Ri-like *Wolbachia*, so we used the phylogram to test whether *Wolbachia* effects on hosts exhibit phylogenetic signal. First, we treated *Wolbachia* effects on host locomotor activity as a binary trait and tested for phylogenetic signal using the *D* statistic [72], implemented in the *caper* R package [73]. Second, we treated *Wolbachia* effects on activity as a continuous trait and tested for phylogenetic signal using Pagel’s lambda (λ) [74]. Here, we analyzed each sex separately, because we found significant infection-by-sex interaction effects on activity (Tables S2 and S3). For each sex, we extracted the least-square (LS) mean ADS for infected and uninfected flies from the linear models (Tables S2 and S3), and used the change in LS mean activity as a continuous character to calculate the maximum likelihood value of Pagel’s λ [74,75]. We used a likelihood ratio test to compare our fitted value of λ to a model assuming no phylogenetic signal ($\lambda = 0$) using the “phylosig” function in the R package *phytools* [76].

RESULTS

Wolbachia infections modify host locomotor activity

We assayed the locomotor activity of 3,104 flies (Figure 1). *Wolbachia* had a significant effect on the activity of six host genotypes, including hosts infected with both A- and B-group

Wolbachia. Interestingly, the direction of *Wolbachia* effects on host activity varied by genotype and sex (Figure 2). We found a significant *Wolbachia* infection-by-sex interaction effect for the *wMelCS-D. melanogaster* genotype that increased male activity ($F = 4.566$, $P = 0.033$; Table S3). We also found a significant infection-by-sex effect for the *wRi-D. simulans* genotype, but *Wolbachia* increased female activity ($F = 8.150$, $P = 0.005$; Table S2). The two other closely related *wRi*-like *Wolbachia*, *wSuz* and *wAur*, also had significant effects on host activity. The *wSuz-D. suzukii* genotype had a significant main effect of *Wolbachia* that reduced host activity ($F = 11.311$, $P < 0.001$; Table S2), and the *wAur-D. auraria* genotype had a significant infection-by-sex interaction that reduced female activity ($F = 6.584$, $P = 0.011$; Table S2). The *wHa-D. simulans* genotype had a significant main effect of *Wolbachia* that increased host activity ($F = 7.764$, $P = 0.006$; Table S2). Lastly, we found the *wHa-wNo* co-infected *D. simulans* genotype had a significant infection-by-sex interaction effect that reduced male activity ($F = 7.076$, $P = 0.008$; Table S2). Because this genotype is co-infected, we do not know the relative contributions of *wHa* and *wNo* to variation in host activity. See the Supplemental Results for a discussion of how other variables contributed to variation in locomotor activity.

Limited evidence for phylogenetic signal

We estimated a Bayesian phylogram of A- and B-group *Wolbachia* using 211 single-copy genes of identical length in all *Wolbachia* genomes, spanning 178,569 bp (Figure 2). We then tested whether closely related *Wolbachia* have similar effects on host activity. When treating *Wolbachia* effects on activity as a binary trait, our estimate of $D = 0.322$ was low, but not statistically different from a model of $D = 1$ assuming phylogenetic randomness ($P = 0.101$) or a model of $D = 0$ with strong phylogenetic signal ($P = 0.198$). Simulations of similar phylogenies with an increasing number of *Wolbachia* strains suggest that at least $N = 50$ strains are required to differentiate our estimated value of $D = 0.322$ from a model of phylogenetic randomness ($D = 1$) (Figures S4 and S5). Thus, *Wolbachia* effects on host activity may exhibit phylogenetic signal, but many more *Wolbachia* strains are required to test this hypothesis. Unfortunately, $N = 50$ strains are not presently available in culture. We also treated *Wolbachia* changes to host activity as a continuous trait; however, we found that maximum likelihood fitted λ values were extremely low, indicative of no phylogenetic signal. λ values generated from the LS mean log-transformed ADS data were not statistically different from zero for females ($\lambda < 0.001$, $P = 1$) or

males ($\lambda < 0.001$, $P = 1$). This was also true when we repeated the analyses for the LS mean square root-transformed ADS data for females ($\lambda < 0.001$, $P = 1$) and males ($\lambda < 0.001$, $P = 1$).

DISCUSSION

Our analyses suggest that *Wolbachia* commonly alter host locomotor activity, which may affect host fitness. Locomotion is a basic host activity underlying many ecologically important behaviors, including foraging, thermoregulation, and mate seeking. In combination with our recent work demonstrating pervasive effects of A- and B-group *Wolbachia* on host temperature preference [20], we posit that *Wolbachia* infections may often alter host behavior.

The wRi-like *Wolbachia* strains in our study (wRi, wSuz, and wAur) consistently altered host activity. We found a low, but non-significant *D* value of 0.322, suggesting effects on host activity may exhibit phylogenetic signal; although, an excessive number of *Wolbachia* strains are required to test this hypothesis. Our findings are consistent with prior experiments demonstrating that wRi increased female *D. simulans* activity in response to olfactory cues [77,78]. We hypothesize that the prominence of wRi-like *Wolbachia* effects on host activity relative to other strains may be due to variation in *Wolbachia* tissue localization [49,52]. wRi occurs at high titer in adult *D. simulans* brains and localizes to specific regions, whereas wMel shows a relatively even distribution in *D. melanogaster* [52]. wRi also occurs at higher titer in the ventral nerve cord, which is a major neural circuit center for motor activities such as walking [52,79–81]. Future experiments should compare *Wolbachia* titer and localization in adult brains for wRi-like variants and strains that do not alter locomotor activity.

We also found considerable variation in the direction and sex-bias of *Wolbachia* effects on locomotor activity (Figure 2). *Wolbachia* decreased activity for wSuz, wAur, and the wNo-wHa co-infection, whereas wMelCS, wRi, and wHa increased activity. These effects were female-biased for wRi and wAur, but male-biased for wMelCS and wNo-wHa. This variation had no relationship to the *Wolbachia* phylogeny, because we found no evidence for phylogenetic signal when measuring *Wolbachia* effects on females and males as a continuous trait ($\lambda < 0.001$). Specific *Wolbachia* effects on host activity may depend on interactions with the host background. For example, our work and others' suggests that identical wMelCS variants have different effects on *D. melanogaster* temperature preference depending on the host background [20,57,58]. Host genomes also modify *Wolbachia* titer [82], *Wolbachia* maternal transmission

[83], components of host fitness [84–87], and the strength of cytoplasmic incompatibility [88–90].

Changes to host activity could underlie *Wolbachia*-induced behaviors that promote infection spread. For example, *wMel*-infected *D. melanogaster* have higher field recapture rates than uninfected flies [91], and long distance dispersal of the spider *Erigone atra* is altered by *Rickettsia*, an endosymbiont closely related to *Wolbachia* [92]. Our own work suggests *Wolbachia* may alter host temperature preference to promote *Wolbachia* replication within host bodies [20]. Other experiments suggest that *wMel* and *wRi* may influence male mating rate [93,94]. Alternatively, hosts may be modifying their own behavior as a response to *Wolbachia* infection. Several studies indicate that *wMel* alters circadian activity and sleep patterns of *D. melanogaster* [52,54–56]. For example, Bi et al. [55] report that *wMel* increases sleep time, which could represent a host immune response to infection [19]. Ultimately, these effects on host behavior factor into how *Wolbachia* influence host fitness, which determines the spread and persistence of *Wolbachia* in host populations [4,95–98]. Because locomotor activity is such a fundamental host behavior, our results suggest *Wolbachia* may have complex and variable effects on many components of host fitness.

ACKNOWLEDEMENTS

We thank TBW for lab assistance and WRC for help with bioinformatic analyses. The Cooper lab group and MT provided valuable feedback that improved the manuscript. This work was supported by NIGMS of the NIH under award R35GM124701 to BSC.

DATA AVAILABILITY

All data and code are available on Dryad (doi:10.5061/dryad.6t1g1jwxv; temporary URL: <https://datadryad.org/stash/share/D4Yi8zYFchXE-LIFgTc6S91s2Nl5Afcwu4-FkxJZPaA>). Genome assemblies will be deposited on GenBank upon acceptance.

FIGURE LEGENDS

Figure 1. Activity of uninfected and infected flies for each sex of each genotype. Activity is measured as mean absolute distance sums (ADS). Significance was evaluated using linear models (Tables S2 and S3).

Figure 2. (A) Estimated Bayesian phylogram for A- and B-group *Wolbachia* strains. The divergence estimate for A- and B-groups is superimposed from Meany et al. [59]. All nodes have Bayesian posterior probabilities of 1. **(B)** *Wolbachia* effects on host activity scored as a binary trait: *Wolbachia* significantly altered host activity (black circle) or had no effect (white circle). **(C)** *Wolbachia* effects on activity scored as a continuous trait: the change in least-square (LS) mean log-transformed activity (ADS) for each sex. LS means were generated from linear models (Table S2). LS mean square root-transformed ADS data are shown in Figure S3.

REFERENCES

1. Moran NA, McCutcheon JP, Nakabachi A. 2008 Genomics and evolution of heritable bacterial symbionts. *Annual Review of Genetics* **42**, 165–190.
2. McFall-Ngai M *et al.* 2013 Animals in a bacterial world, a new imperative for the life sciences. *Proceedings of the National Academy of Sciences* **110**, 3229–3236.
3. McCutcheon JP, Boyd BM, Dale C. 2019 The life of an insect endosymbiont from the cradle to the grave. *Current Biology* **29**, R485–R495.
4. Hoffmann AA, Turelli M. 1997 Cytoplasmic incompatibility in insects. In *Influential passengers: inherited microorganisms and arthropod reproduction* (eds SL O'Neill, AA Hoffmann, JH Werren), pp. 42–80. Oxford University Press.
5. Werren JH, Baldo L, Clark ME. 2008 *Wolbachia*: master manipulators of invertebrate biology. *Nature Reviews Microbiology* **6**, 741–751.
6. Hurst G, Hurst L, Majerus M. 1997 Cytoplasmic sex-ratio distorters. In *Influential passengers: inherited microorganisms and arthropod reproduction* (eds SL O'Neill, AA Hoffmann, JH Werren), pp. 125–154. Oxford University Press.
7. Shropshire JD, Leigh B, Bordenstein SR. 2020 Symbiont-mediated cytoplasmic incompatibility: what have we learned in 50 years? *Elife* **9**, e61989.
8. Baumann P. 2005 Biology of bacteriocyte-associated endosymbionts of plant sap-sucking insects. *Annual Review of Microbiology* **59**, 155–189.
9. Douglas AE. 2009 The microbial dimension in insect nutritional ecology. *Functional Ecology* **23**, 38–47.
10. Brumin M, Kontsedalov S, Ghanim M. 2011 *Rickettsia* influences thermotolerance in the whitefly *Bemisia tabaci* B biotype. *Insect Science* **18**, 57–66.
11. Mueller UG *et al.* 2011 Evolution of cold-tolerant fungal symbionts permits winter fungiculture by leafcutter ants at the northern frontier of a tropical ant–fungus symbiosis. *Proceedings of the National Academy of Sciences* **108**, 4053–4056.
12. Hedges LM, Brownlie JC, O'Neill SL, Johnson KN. 2008 *Wolbachia* and virus protection in insects. *Science* **322**, 702–702.
13. Teixeira L, Ferreira Á, Ashburner M. 2008 The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. *PLoS Biology* **6**, e1000002.
14. Jaenike J, Unckless R, Cockburn SN, Boelio LM, Perlman SJ. 2010 Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. *Science* **329**, 212–215.

- 241 15. Oliver KM, Smith AH, Russell JA. 2014 Defensive symbiosis in the real world—advancing
242 ecological studies of heritable, protective bacteria in aphids and beyond. *Functional*
243 *Ecology* **28**, 341–355.
- 244 16. Feldhaar H. 2011 Bacterial symbionts as mediators of ecologically important traits of insect
245 hosts. *Ecological Entomology* **36**, 533–543.
- 246 17. Goodacre SL, Martin OY. 2012 Modification of insect and arachnid behaviours by
247 vertically transmitted endosymbionts: infections as drivers of behavioural change and
248 evolutionary novelty. *Insects* **3**, 246–261.
- 249 18. Schretter CE, Vielmetter J, Bartos I, Marka Z, Marka S, Argade S, Mazmanian SK. 2018 A
250 gut microbial factor modulates locomotor behaviour in *Drosophila*. *Nature* **563**, 402–406.
- 251 19. Bi J, Wang Y-F. 2020 The effect of the endosymbiont *Wolbachia* on the behavior of insect
252 hosts. *Insect Science* **27**, 846–858.
- 253 20. Hague MT, Caldwell CN, Cooper BS. 2020 Pervasive effects of *Wolbachia* on host
254 temperature preference. *mBio* **11**.
- 255 21. Hosokawa T, Fukatsu T. 2020 Relevance of microbial symbiosis to insect behavior.
256 *Current Opinion in Insect Science* **39**, 91–100.
- 257 22. Thomas F, Adamo S, Moore J. 2005 Parasitic manipulation: where are we and where
258 should we go? *Behavioural Processes* **68**, 185–199.
- 259 23. Lefevre T, Thomas F. 2008 Behind the scene, something else is pulling the strings:
260 emphasizing parasitic manipulation in vector-borne diseases. *Infection, Genetics and*
261 *Evolution* **8**, 504–519.
- 262 24. Poulin R. 2010 Parasite manipulation of host behavior: an update and frequently asked
263 questions. In *Advances in the Study of Behavior*, pp. 151–186. Elsevier.
- 264 25. van Houte S, Ros VI, van Oers MM. 2013 Walking with insects: molecular mechanisms
265 behind parasitic manipulation of host behaviour. *Molecular Ecology* **22**, 3458–3475.
- 266 26. Heil M. 2016 Host manipulation by parasites: cases, patterns, and remaining doubts.
267 *Frontiers in Ecology and Evolution* **4**, 80.
- 268 27. Vale PF, Siva-Jothy A, Morrill A, Forbes MR. 2018 The influence of parasites. In *Insect*
269 *behavior: from mechanisms to ecological and evolutionary consequences*, pp. 273–291.
270 Oxford University Press Oxford.
- 271 28. Weinersmith KL. 2019 What’s gotten into you?: A review of recent research on parasitoid
272 manipulation of host behavior. *Current Opinion in Insect Science*

- 273 29. Louis C, Jourdan M, Cabanac M. 1986 Behavioral fever and therapy in a rickettsia-infected
274 Orthoptera. *American Journal of Physiology-Regulatory, Integrative and Comparative*
275 *Physiology* **250**, R991–R995.
- 276 30. Fedorka KM, Kutch IC, Collins L, Musto E. 2016 Cold temperature preference in
277 bacterially infected *Drosophila melanogaster* improves survival but is remarkably
278 suboptimal. *Journal of Insect Physiology* **93**, 36–41.
- 279 31. Adelman JS, Martin LB. 2009 Vertebrate sickness behaviors: adaptive and integrated
280 neuroendocrine immune responses. *Integrative and Comparative Biology* **49**, 202–214.
- 281 32. Opp MR. 2009 Sleeping to fuel the immune system: mammalian sleep and resistance to
282 parasites. *BMC Evolutionary Biology* **9**, 1–3.
- 283 33. Kuo T-H, Pike DH, Beizaeipour Z, Williams JA. 2010 Sleep triggered by an immune
284 response in *Drosophila* is regulated by the circadian clock and requires the NFκB Relish.
285 *BMC Neuroscience* **11**, 1–12.
- 286 34. Kuo T-H, Williams JA. 2014 Increased sleep promotes survival during a bacterial infection
287 in *Drosophila*. *Sleep* **37**, 1077–1086.
- 288 35. Zug R, Hammerstein P. 2012 Still a host of hosts for *Wolbachia*: Analysis of recent data
289 suggests that 40% of terrestrial arthropod species are infected. *PLoS One* **7**, e38544.
- 290 36. Weinert LA, Araujo-Jnr EV, Ahmed MZ, Welch JJ. 2015 The incidence of bacterial
291 endosymbionts in terrestrial arthropods. *Proceedings of the Royal Society B: Biological*
292 *Sciences* **282**, 20150249.
- 293 37. Bandi C, Anderson TJ, Genchi C, Blaxter ML. 1998 Phylogeny of *Wolbachia* in filarial
294 nematodes. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **265**,
295 2407–2413.
- 296 38. O'Neill SL, Giordano R, Colbert AM, Karr TL, Robertson HM. 1992 16S rRNA
297 phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic
298 incompatibility in insects. *Proceedings of the National Academy of Sciences* **89**, 2699–
299 2702.
- 300 39. Raychoudhury R, Baldo L, Oliveira DC, Werren JH. 2009 Modes of acquisition of
301 *Wolbachia*: Horizontal transfer, hybrid introgression, and codivergence in the *Nasonia*
302 species complex. *Evolution* **63**, 165–183.
- 303 40. Conner WR, Blaxter ML, Anfora G, Ometto L, Rota-Stabelli O, Turelli M. 2017 Genome
304 comparisons indicate recent transfer of wRi-like *Wolbachia* between sister species
305 *Drosophila suzukii* and *D. subpulchrella*. *Ecology and Evolution* **7**, 9391–9404.
- 306 41. Gerth M, Bleidorn C. 2017 Comparative genomics provides a timeframe for *Wolbachia*
307 evolution and exposes a recent biotin synthesis operon transfer. *Nature Microbiology* **2**,
308 16241.

- 309 42. Turelli M *et al.* 2018 Rapid global spread of *w*Ri-like *Wolbachia* across multiple
310 *Drosophila*. *Current Biology* **28**, 963–971.e8.
- 311 43. Cooper BS, Vanderpool D, Conner WR, Matute DR, Turelli M. 2019 *Wolbachia*
312 acquisition by *Drosophila yakuba*-clade hosts and transfer of incompatibility loci between
313 distantly related *Wolbachia*. *Genetics* **212**, 1399–1419.
- 314 44. Prout T. 1994 Some evolutionary possibilities for a microbe that causes incompatibility in
315 its host. *Evolution* **48**, 909–911.
- 316 45. Turelli M. 1994 Evolution of incompatibility-inducing microbes and their hosts. *Evolution*
317 **48**, 1500–1513.
- 318 46. Weeks AR, Turelli M, Harcombe WR, Reynolds KT, Hoffmann AA. 2007 From parasite to
319 mutualist: Rapid evolution of *Wolbachia* in natural populations of *Drosophila*. *PLoS*
320 *Biology* **5**, e114.
- 321 47. Haygood R, Turelli M. 2009 Evolution of incompatibility-inducing microbes in subdivided
322 host populations. *Evolution* **63**, 432–447.
- 323 48. Dobson SL, Bourtzis K, Braig HR, Jones BF, Zhou W, Rousset F, O'Neill SL. 1999
324 *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. *Insect*
325 *Biochemistry and Molecular Biology* **29**, 153–160.
- 326 49. Albertson R, Casper-Lindley C, Cao J, Tram U, Sullivan W. 2009 Symmetric and
327 asymmetric mitotic segregation patterns influence *Wolbachia* distribution in host somatic
328 tissue. *Journal of Cell Science* **122**, 4570–4583.
- 329 50. Casper-Lindley C, Kimura S, Saxton DS, Essaw Y, Simpson I, Tan V, Sullivan W. 2011
330 Rapid fluorescence-based screening for *Wolbachia* endosymbionts in *Drosophila* germ line
331 and somatic tissues. *Applied Environmental Microbiology* **77**, 4788–4794.
- 332 51. Pietri JE, DeBruhl H, Sullivan W. 2016 The rich somatic life of *Wolbachia*.
333 *MicrobiologyOpen* **5**, 923–936.
- 334 52. Albertson R, Tan V, Leads RR, Reyes M, Sullivan W, Casper-Lindley C. 2013 Mapping
335 *Wolbachia* distributions in the adult *Drosophila* brain. *Cellular Microbiology* **15**, 1527–
336 1544.
- 337 53. Fleury F, Vavre F, Ris N, Fouillet P, Bouletreau M. 2000 Physiological cost induced by the
338 maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid *Leptopilina*
339 *heterotoma*. *Parasitology* **121**, 493–500.
- 340 54. Vale PF, Jardine MD. 2015 Sex-specific behavioural symptoms of viral gut infection and
341 *Wolbachia* in *Drosophila melanogaster*. *Journal of Insect Physiology* **82**, 28–32.
- 342 55. Bi J, Sehgal A, Williams JA, Wang Y-F. 2018 *Wolbachia* affects sleep behavior in
343 *Drosophila melanogaster*. *Journal of Insect Physiology* **107**, 81–88.

56. Morioka E, Oida M, Tsuchida T, Ikeda M. 2018 Nighttime activities and peripheral clock oscillations depend on *Wolbachia* endosymbionts in flies. *Scientific reports* **8**, 15432.
57. Arnold PA, Levin SC, Stevanovic AL, Johnson KN. 2019 *Drosophila melanogaster* infected with *Wolbachia* strain wMelCS prefer cooler temperatures. *Ecological Entomology* **44**, 287–290.
58. Truitt AM, Kapun M, Kaur R, Miller WJ. 2019 *Wolbachia* modifies thermal preference in *Drosophila melanogaster*. *Environmental Microbiology* **21**, 3259–3268.
59. Meany MK, Conner WR, Richter SV, Bailey JA, Turelli M, Cooper BS. 2019 Loss of cytoplasmic incompatibility and minimal fecundity effects explain relatively low *Wolbachia* frequencies in *Drosophila mauritiana*. *Evolution* **73**, 1278–1295.
60. O'Neill SL, Karr TL. 1990 Bidirectional incompatibility between conspecific populations of *Drosophila simulans*. *Nature* **348**, 178–180.
61. Mercot H, Llorente B, Jacques M, Atlan A, Montchamp-Moreau C. 1995 Variability within the Seychelles cytoplasmic incompatibility system in *Drosophila simulans*. *Genetics* **141**, 1015–1023.
62. Rousset F, Solignac M. 1995 Evolution of single and double *Wolbachia* symbioses during speciation in the *Drosophila simulans* complex. *Proceedings of the National Academy of Sciences* **92**, 6389–6393.
63. James A, Dean M, McMahon M, Ballard J. 2002 Dynamics of double and single *Wolbachia* infections in *Drosophila simulans* from New Caledonia. *Heredity* **88**, 182–189.
64. Ballard J, Melvin R. 2007 Tetracycline treatment influences mitochondrial metabolism and mtDNA density two generations after treatment in *Drosophila*. *Insect Molecular Biology* **16**, 799–802.
65. Videlier M, Rundle HD, Careau V. 2019 Sex-specific among-individual covariation in locomotor activity and resting metabolic rate in *Drosophila melanogaster*. *The American Naturalist* **194**, E164–E176.
66. Videlier M, Careau V, Wilson AJ, Rundle HD. 2021 Quantifying selection on standard metabolic rate and body mass in *Drosophila melanogaster*. *Evolution* **75**, 130–140.
67. Fox J, Weisberg S. 2019 *An R Companion to Applied Regression*. Third. Thousand Oaks CA: Sage. See <https://socialsciences.mcmaster.ca/jfox/Books/Companion/>.
68. R Core Team. 2018 *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. See <https://www.R-project.org/>.
69. Klasson L *et al.* 2009 The mosaic genome structure of the *Wolbachia* wRi strain infecting *Drosophila simulans*. *Proceedings of the National Academy of Sciences* **106**, 5725–5730.

- 378 70. Ellegaard KM, Klasson L, Näslund K, Bourtzis K, Andersson SG. 2013 Comparative
379 genomics of *Wolbachia* and the bacterial species concept. *PLoS Genetics* **9**.
- 380 71. Siozios S, Cestaro A, Kaur R, Pertot I, Rota-Stabelli O, Anfora G. 2013 Draft genome
381 sequence of the *Wolbachia* endosymbiont of *Drosophila suzukii*. *Genome announcements*
382 **1**, e00032-13.
- 383 72. Fritz SA, Purvis A. 2010 Selectivity in mammalian extinction risk and threat types: a new
384 measure of phylogenetic signal strength in binary traits. *Conservation Biology* **24**, 1042–
385 1051.
- 386 73. Orme D, Freckleton R, Thomas G, Petzoldt T, Fritz S, Isaac N, Pearse W. 2018 *caper*:
387 *Comparative Analyses of Phylogenetics and Evolution in R*. See [https://CRAN.R-](https://CRAN.R-project.org/package=caper)
388 [project.org/package=caper](https://CRAN.R-project.org/package=caper).
- 389 74. Pagel M. 1999 Inferring the historical patterns of biological evolution. *Nature* **401**, 877–
390 884.
- 391 75. Freckleton RP, Harvey PH, Pagel M. 2002 Phylogenetic analysis and comparative data: a
392 test and review of evidence. *The American Naturalist* **160**, 712–726.
- 393 76. Revell LJ. 2012 phytools: an R package for phylogenetic comparative biology (and other
394 things). *Methods in Ecology and Evolution* **3**, 217–223.
- 395 77. Peng Y, Nielsen JE, Cunningham JP, McGraw EA. 2008 *Wolbachia* infection alters
396 olfactory-cued locomotion in *Drosophila* spp. *Applied Environmental Microbiology* **74**,
397 3943–3948.
- 398 78. Peng Y, Wang Y. 2009 Infection of *Wolbachia* may improve the olfactory response of
399 *Drosophila*. *Chinese Science Bulletin* **54**, 1369–1375.
- 400 79. Burrows M, Laurent G, Field L. 1988 Proprioceptive inputs to nonspiking local
401 interneurons contribute to local reflexes of a locust hindleg. *Journal of Neuroscience* **8**,
402 3085–3093.
- 403 80. Laurent G, Burrows M. 1988 A population of ascending intersegmental interneurons in the
404 locust with mechanosensory inputs from a hind leg. *Journal of Comparative Neurology*
405 **275**, 1–12.
- 406 81. Yellman C, Tao H, He B, Hirsh J. 1997 Conserved and sexually dimorphic behavioral
407 responses to biogenic amines in decapitated *Drosophila*. *Proceedings of the national*
408 *academy of sciences* **94**, 4131–4136.
- 409 82. Funkhouser-Jones LJ, van Opstal EJ, Sharma A, Bordenstein SR. 2018 The maternal effect
410 gene Wds controls *Wolbachia* titer in *Nasonia*. *Current Biology* **28**, 1692–1702.
- 411 83. Serbus LR, Sullivan W. 2007 A cellular basis for *Wolbachia* recruitment to the host
412 germline. *PLoS Pathogens* **3**, e190.

- 413 84. Fry A, Palmer M, Rand D. 2004 Variable fitness effects of *Wolbachia* infection in
414 *Drosophila melanogaster*. *Heredity* **93**, 379.
- 415 85. Dean MD. 2006 A *Wolbachia*-associated fitness benefit depends on genetic background in
416 *Drosophila simulans*. *Proceedings of the Royal Society of London B: Biological Sciences*
417 **273**, 1415–1420.
- 418 86. Gruntenko NE, Karpova EK, Adonyeva NV, Andreenkova OV, Burdina EV, Ilinsky YY,
419 Bykov RA, Menshanov PN, Rauschenbach IY. 2019 *Drosophila* female fertility and
420 juvenile hormone metabolism depends on the type of *Wolbachia* infection. *Journal of*
421 *Experimental Biology* **222**, jeb195347.
- 422 87. Serga SV, Maistrenko OM, Matiytsiv NP, Vaiserman AM, Kozeretska IA. 2021 Effects of
423 *Wolbachia* infection on fitness-related traits in *Drosophila melanogaster*. *Symbiosis*
424 (doi:10.1007/s13199-020-00743-3)
- 425 88. Reynolds KT, Hoffmann AA. 2002 Male age, host effects and the weak expression or non-
426 expression of cytoplasmic incompatibility in *Drosophila* strains infected by maternally
427 transmitted *Wolbachia*. *Genetics Research* **80**, 79–87.
- 428 89. Cooper BS, Ginsberg PS, Turelli M, Matute DR. 2017 *Wolbachia* in the *Drosophila yakuba*
429 complex: Pervasive frequency variation and weak cytoplasmic incompatibility, but no
430 apparent effect on reproductive isolation. *Genetics* **205**, 333–351.
- 431 90. Cattel J *et al.* 2018 Back and forth *Wolbachia* transfers reveal efficient strains to control
432 spotted wing *Drosophila* populations. *Journal of Applied Ecology* **55**, 2408–2418.
- 433 91. Caragata EP, Real KM, Zalucki MP, McGraw EA. 2011 *Wolbachia* infection increases
434 recapture rate of field-released *Drosophila melanogaster*. *Symbiosis* **54**, 55.
- 435 92. Goodacre SL, Martin OY, Bonte D, Hutchings L, Woolley C, Ibrahim K, Thomas CG,
436 Hewitt GM. 2009 Microbial modification of host long-distance dispersal capacity. *BMC*
437 *Biology* **7**, 1–8.
- 438 93. De Crespigny FC, Pitt T, Wedell N. 2006 Increased male mating rate in *Drosophila* is
439 associated with *Wolbachia* infection. *Journal of Evolutionary Biology* **19**, 1964–1972.
- 440 94. De Crespigny FEC, Wedell N. 2007 Mate preferences in *Drosophila* infected with
441 *Wolbachia*? *Behavioral Ecology and Sociobiology* **61**, 1229.
- 442 95. Hoffmann AA, Turelli M, Harshman LG. 1990 Factors affecting the distribution of
443 cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* **126**, 933–948.
- 444 96. Kriesner P, Hoffmann AA. 2018 Rapid spread of a *Wolbachia* infection that does not affect
445 host reproduction in *Drosophila simulans* cage populations. *Evolution* **72**, 1475–1487.
- 446 97. Ross PA, Turelli M, Hoffmann AA. 2019 Evolutionary ecology of *Wolbachia* releases for
447 disease control. *Annual Review of Genetics* **53**.

98. Kriesner P, Conner WR, Weeks AR, Turelli M, Hoffmann AA. 2016 Persistence of a
Wolbachia infection frequency cline in *Drosophila melanogaster* and the possible role of
reproductive dormancy. *Evolution* **70**, 979–997.

A **B**