© 2024. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2024) 227, jeb247168. doi:10.1242/jeb.247168

RESEARCH ARTICLE

Wolbachia-infected pharaoh ant colonies have higher egg production, metabolic rate and worker survival

Rohini Singh[1], Sachin Suresh[2], Jennifer H. Fewell[2], Jon F. Harrison[2] and Timothy A. Linksvayer[1,2,*]

ABSTRACT

Wolbachia is a widespread maternally transmitted endosymbiotic bacteria with diverse phenotypic effects on its insect hosts, ranging from parasitic to mutualistic. *Wolbachia* commonly infects social insects, where it faces unique challenges associated with its host's caste-based reproductive division of labor and colony living. Here, we dissect the benefits and costs of *Wolbachia* infection on life-history traits of the invasive pharaoh ant, *Monomorium pharaonis*, which are relatively short lived and show natural variation in *Wolbachia* infection status between colonies. We quantified the effects of *Wolbachia* infection on the lifespan of queen and worker castes, the egg-laying rate of queens across queen lifespan, and the metabolic rates of whole colonies and colony members. Infected queens laid more eggs than uninfected queens but had similar metabolic rates and lifespans. Interestingly, infected workers outlived uninfected workers. At the colony level, infected colonies were more productive as a consequence of increased queen egg-laying rates and worker longevity, and infected colonies had higher metabolic rates during peak colony productivity. While some effects of infection, such as elevated colony-level metabolic rates, may be detrimental in more stressful natural conditions, we did not find any costs of infection under relatively benign laboratory conditions. Overall, our study emphasizes that *Wolbachia* infection can have beneficial effects on ant colony growth and worker survival in at least some environments.

KEY WORDS: Life history, Trade-offs, Endosymbiont, Eusocial insects

INTRODUCTION

Wolbachia, a widespread maternally transmitted endosymbiotic bacteria, is best known for its ability to manipulate a wide range of hosts (Ramalho et al., 2021; Russell et al., 2009). *Wolbachia* is estimated to have infected more than 65% of all insect species but is also widespread in other invertebrates such as crustaceans, arachnids and nematodes (Werren et al., 2008). This bacterium often manipulates the host reproductive systems, thereby causing cytoplasmic incompatibility between infected and uninfected mates, killing or feminizing infected males, causing female-biased sex ratios or inducing parthenogenesis (Engelstädter and Hurst, 2009; Shoemaker et al., 1999; Zug and Hammerstein, 2015). *Wolbachia* can also have fitness-enhancing effects for the host, such as increased host fecundity and survival, conditional on the *Wolbachia* strain, host genotype, host species and environment (Fry et al., 2004; Gruntenko et al., 2017, 2019; Mouton et al., 2007; Reynolds et al., 2003; White et al., 2011; Zélé et al., 2020).

Even though *Wolbachia* infects an estimated one-third of all ant species (Lee et al., 2020; Russell, 2012; Tseng et al., 2019), we have a limited understanding of the phenotypic effects of *Wolbachia* on ants and other social insects (Moreau, 2020; Russell, 2012). As in solitary insects, *Wolbachia* has often been considered to be a manipulator of reproductive strategies in multiple species of ants (Shoemaker et al., 2000; Van Borm et al., 2001; Wenseleers et al., 1998, 2002). For instance, *Wolbachia*-infected species are more likely to have dependent colony foundation, where two or more queens start a nest site together, which is associated with changes in patterns of colony-level resource investment and queen phenotypes (Treanor and Hughes, 2019). In the ant *Formica truncorum*, infected colonies produced significantly fewer new queens and males, indicating *Wolbachia* can have a deleterious effect in some cases (Wenseleers et al., 2002). Recently, it has been shown that colony aggression in *Anoplolepis gracilipes* is correlated with *Wolbachia* prevalence and temperature (Lin et al., 2023). Overall, when compared with solitary insects, *Wolbachia*-induced effects in eusocial insects can differ as a result of their distinct biology, including reproductive division of labor and obligately cooperative lifestyle (Bouwma et al., 2006; de Bekker et al., 2018; Kautz et al., 2013; Lin et al., 2023; Reuter et al., 2005; Rey et al., 2013; Russell, 2012; Shoemaker et al., 2000; Tsutsui et al., 2003; Wenseleers et al., 1998, 2002).

We previously showed that colonies of the invasive pharaoh ant *Monomorium pharaonis* that are infected by *Wolbachia* have higher colony growth, measured in terms of the production of new workers and queens (Singh and Linksvayer, 2020), as well as queen-biased sex ratios (Pontieri et al., 2017; Singh and Linksvayer, 2020) under laboratory conditions. In the current study, we sought to further elucidate the costs and benefits of *Wolbachia* infection in *M. pharaonis* colonies. As overall colony growth rates are determined by 'birth' rates (i.e. queen egg-laying rates) and death rates (i.e. lifespans of adult workers and queens), we specifically quantified the effects of *Wolbachia* on queen fecundity and the lifespan of queens and workers. Because effects of *Wolbachia* on queen fecundity and colony productivity might also be expected to lead to metabolic costs, we also quantified effects of *Wolbachia* infection on the metabolic rate of colonies. Overall, we sought to test whether higher colony-level productivity of *Wolbachia*-infected colonies (Singh and Linksvayer, 2020) is explained by higher egg laying of infected queens and/or effects on queen or worker lifespans, and whether infection by *Wolbachia* has any detectable costs in terms of increased metabolism and decreased queen and/or worker lifespan.

MATERIALS AND METHODS

Source of infected and uninfected *Monomorium pharaonis* colonies and ant husbandry

To initially produce a population of *Monomorium pharaonis* (Linnaeus 1758) colonies that differed in terms of *Wolbachia*

[1]Department of Biology, University of Pennsylvania, Philadelphia, PA 19104, USA. [2]School of Life Sciences, Arizona State University, Tempe, AZ 85287, USA.

*Author for correspondence (timothy.linksvayer@asu.edu)

R.S., 0000-0003-0360-374X; S.S., 0000-0002-5245-7008; J.H.F., 0000-0003-2124-5608; J.F.H., 0000-0001-5223-216X; T.A.L., 0000-0001-7034-1546

Received 2 January 2024; Accepted 18 July 2024

Journal of Experimental Biology (2024) 227, jeb247168. doi:10.1242/jeb.247168

infection status, where genetic background and infection status were relatively uncoupled, we systematically intercrossed colonies that were naturally either infected or uninfected with *Wolbachia* for nine generations (Singh and Linksvayer, 2020). Throughout these generations of systematic intercrossing, we tracked the maternal pedigree of colonies in our laboratory population, and we used this maternal pedigree to predict *Wolbachia* infection status: *Wolbachia*-infected queens are expected to only produce more *Wolbachia*-infected offspring queens and workers, and we previously used a multilocus genotyping assay to verify that *Wolbachia* infection status of colonies is predicted by maternal pedigree (Pontieri et al., 2017; Singh and Linksvayer, 2020). We re-verified the expected infection status of 15 *Wolbachia*-infected stock colonies and 14 uninfected stock colonies (see Supplementary Materials and Methods), and we separately combined these infected and uninfected stock colonies to create two separate pools of ants that differed in *Wolbachia* infection but were genetically homogeneous (Singh and Linksvayer, 2020). We used these two pools to create replicate and genetically homogeneous source colonies of known infection status, and we again confirmed the infection status of these replicate study colonies (see Singh and Linksvayer, 2020).

We experimentally synchronized the age of the queens in these source colonies by removing all existing adult queens from the colonies to initiate the production of new virgin queens and males and restart the colony life cycle. This produced queens of known and the same age across all the source colonies. These queen age-matched source colonies with known infection status were then used as sources for known-aged queens and to create replicate experimental colonies for all the assays performed below. All colonies used in the current study were reared at 27±1°C with approximately 50% relative humidity, and fed *ad libitum* synthetic agar diet (sugar:protein=3:1; Dussutour and Simpson, 2008) and dried mealworms (*Tenebrio molitor*) twice a week.

Egg laying by newly mated queens across 50 days

We first compared the numbers of eggs produced by newly mated infected and uninfected queens over 50 days in replicate experimental colonies. These replicate experimental colonies were each created with 50 workers and 20 virgin queens that were mated with 15 virgin males. We lightly anesthetized the colonies with CO_2 and the number of eggs, larvae, pupae and adults was counted in each colony when the queens were 5, 8, 11, 14, 17, 20, 23, 35, 39, 43 and 50 days old. Any clumps of eggs were gently separated with a paintbrush to facilitate counting the total number of eggs. We used a blind design for the study where the infection status of the experimental colony at the time of the census was unknown.

Egg laying by queens across their lifespan over 9 months

We next compared egg-laying differences of 20 *Wolbachia*-infected and uninfected queens when the queens were 1, 3, 4, 6 and 9 months old to quantify differences across the queens' lifespan (queens usually live 9–12 months; Peacock et al., 1955). We assayed total egg production over a 48 h period by introducing 20 known-aged queens into replicate eggless experimental colonies. Each experimental colony was constructed with approximately 500 adult workers and 500 brood (larvae plus pupae), following a previously described protocol (Singh and Linksvayer, 2020). After 48 h, we counted the total number of eggs in these experimental colonies as a measure of queen egg-laying rate, and then returned the known-aged queens to their respective source colonies.

Effect of *Wolbachia* infection status on the survival of queens

We compared the survival of queens in 18 *Wolbachia*-infected and 16 uninfected small experimental colonies, each initiated with 20 queens that were each 2.5 months old, as well as 50 workers. Once every 3 weeks, and then once a week after 4 months, we censused each colony and counted the number of eggs, larvae, pupae and adults, in particular the number of surviving queens. At each census, we recorded the number of queens within each colony that survived (i.e. individual-level survival) and also whether any queens within each colony survived (i.e. group-level survival). We used a blind design for the study so that the infection status of each colony was unknown while we collected the data.

Effect of *Wolbachia* infection status on the survival of workers

To estimate the effect of *Wolbachia* infection status on worker survival, controlling for worker genotype (see Fig. S2), we compared the survival of 23 groups of 50 *Wolbachia*-infected workers and 25 groups of 50 uninfected workers. We set up these replicate worker groups using newly eclosed adult workers and censused these worker groups once every 3 days, from 30 August 2019 to 2 December 2019. As for queen survival, at each census, we recorded the number of workers within each replicate group that survived (i.e. individual-level survival) and also whether any workers within each replicate group survived (i.e. group-level survival).

Metabolic rate differences between infected and uninfected colonies and colony members

We compared metabolic rates of infected versus uninfected whole colonies at two different colony life stages: colonies with 1 month old queens (n =12) and 3 month old queens (n=8), representative of colonies with young and mature queens, respectively. For the colonies with 1 month old queens, along with quantifying metabolic rates of intact whole colonies, we also estimated metabolic rate of just the brood and just the queens as this is the stage of the colony life cycle (i.e. when queens were 1 month old) when growth curves are steepest (Singh and Linksvayer, 2020) and most likely to be affected by metabolic cost. We estimated metabolic rate using flow-through respirometry (Lighton, 2018) with a LiCor-7000 'differential gas' analyzer for whole colonies (Waters et al., 2010) and brood, and with a LiCor-6252 CO_2 analyzer for groups of 15 queens using the differential gas analyzer mode. We used dry CO_2-free air at a flow rate of 125 ml min^{-1} (25% of 500 ml min^{-1} flow controllers) for whole colonies and brood, and a flow rate of 50 ml min^{-1} (100% of 50 ml min^{-1} flow controllers) for groups of 15 queens. Additional details on respirometry are provided in the Supplementary Materials and Methods and Fig. S1. We used source colonies to create replicate experimental colonies containing queens at the required ages (i.e. 1 or 3 months old).

For the colonies with 1 month old queens, we estimated the metabolic rates of 12 infected and 12 uninfected replicate experimental colonies, with each colony having 20 queens, approximately 250 workers and 250 brood. Metabolic rates of just the brood (i.e. eggs plus larvae plus pre-pupae plus pupae) were estimated from 11 infected and 11 uninfected colonies, after recording from the intact whole colony (i.e. also containing adult workers and queens).

We measured CO_2 emission from one experimental colony per day and alternated between infected and uninfected experimental colonies to ensure that the queens were of similar age between the two groups at the time of measurement. We added a small water tube in the respirometer chamber along with the colony and the brood, to reduce any stress from possible dehydration for the brood. Finally, we also estimated metabolic rates of 14 *Wolbachia*-infected and 15 uninfected

Journal of Experimental Biology (2024) 227, jeb247168. doi:10.1242/jeb.247168

groups of 15 queens that were 1–2 months old. We measured one to four groups of queens per day and alternated between infected and uninfected groups of queens to ensure even sampling across queen ages and colony life cycle stages.

We also estimated the metabolic rates of eight *Wolbachia*-infected and eight uninfected replicate experimental colonies with 3 month old queens, using 20 queens, approximately 500 adult workers and 500 brood (eggs to pupae) per colony. We recorded CO_2 emissions from one infected and one uninfected colony per day. We chose this queen age as *M. pharaonis* colonies peaked in their productivity at this age and *Wolbachia*-infected colonies had increased reproductive investment compared with uninfected colonies (Singh and Linksvayer, 2020). Additional details can be found in the Supplementary Materials and Methods and Fig. S1.

Statistical analysis

We analyzed the data in R version 3.6.1 (http://www.R-project.org/) with *car* (Fox and Weisberg, 2019) and *lme4* (Bates et al., 2015) packages for regression analysis and *ggplot2* (https://CRAN.R-project.org/package=dplyr) for visualization. We used a generalized linear mixed model (GLMM; Bolker et al., 2009) with Poisson error distribution and with *Wolbachia* infection as a fixed factor to quantify effects of *Wolbachia* on per capita queen fecundity over the first 90 days after queens mated. We used a repeated measures design by including colony ID as a random factor. We used a generalized linear model (GLM; Bolker et al., 2009) with negative binomial error distribution and *Wolbachia* infection as a fixed factor to quantify effects of *Wolbachia* infection on per capita queen fecundity across 9 months. For both the GLMM and GLM models, we included queen age (i.e. measured as the number of days in the GLMM and number of months in the GLM) as both a linear and quadratic predictor because queen fecundity is expected to fluctuate periodically as queens age.

We assessed the allometric relationship between metabolic rates of the whole colonies (μW) and mass of the colonies (grams) using a log–log plot (Fig. 3). Metabolic rates were estimated (μW and μW g^{-1} of the experimental group) from CO_2 levels measured in ppm by assuming an oxyjoule of 19.87 J ml^{-1} O_2 (respiratory quotient of 0.75) and standardized to 25°C assuming Q_{10}=2.0 (Lighton, 2018). We used a linear model framework (LM) to test the effects of *Wolbachia* infection, queen age, colony-level activity, colony mass and colony size on estimates of metabolic rate. We computed the test statistic of individual factors in the linear model via ANOVA from the *car* package (Fox and Weisberg, 2019). The dataset used in this study, the detailed R script for data analysis and the output from the regression models are available from Dryad (https://doi.org/10.5061/dryad.0zpc8672b).

RESULTS

Wolbachia-infected pharaoh ant queens are more fecund than uninfected queens

The per capita fecundity of newly mated *Wolbachia*-infected queens across the first 50 days was higher than that of uninfected queens

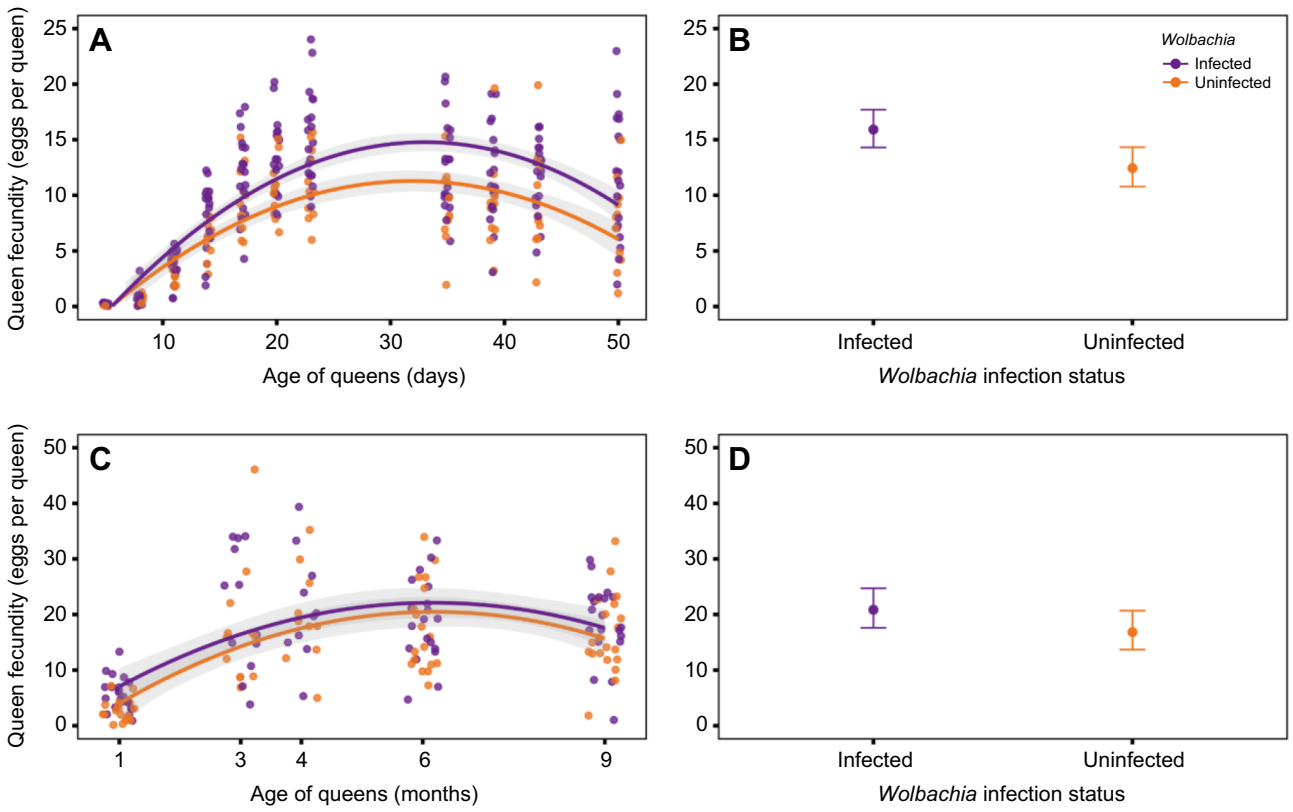

Fig. 1. *Wolbachia*-infected *Monomorium pharaonis* queens have higher fecundity. (A) Groups of newly mated *Wolbachia*-infected queens have higher fecundity than uninfected queens across 50 days. (B) Estimated effect size of *Wolbachia* infection on the fecundity of the queens over 50 days. (C) Groups of *Wolbachia*-infected queens have higher fecundity than uninfected queens across 9 months. (D) Estimated effect size of *Wolbachia* infection on the fecundity of queens over 9 months. For A and C, the *x*-axis represents the age of the queens; for B and D, the *x*-axis represents the *Wolbachia* infection status of the experimental colonies; in all panels, the *y*-axis represents the fecundity of queens (total eggs laid per queen). Data in A and C are observed counts of eggs per queen for each colony (dots), for infected (purple) and uninfected (orange) colonies. Predicted eggs per queen across colonies from a GLMM (A) and GLM (C) are shown by lines with 95% confidence intervals (shaded area).

Journal of Experimental Biology (2024) 227, jeb247168. doi:10.1242/jeb.247168

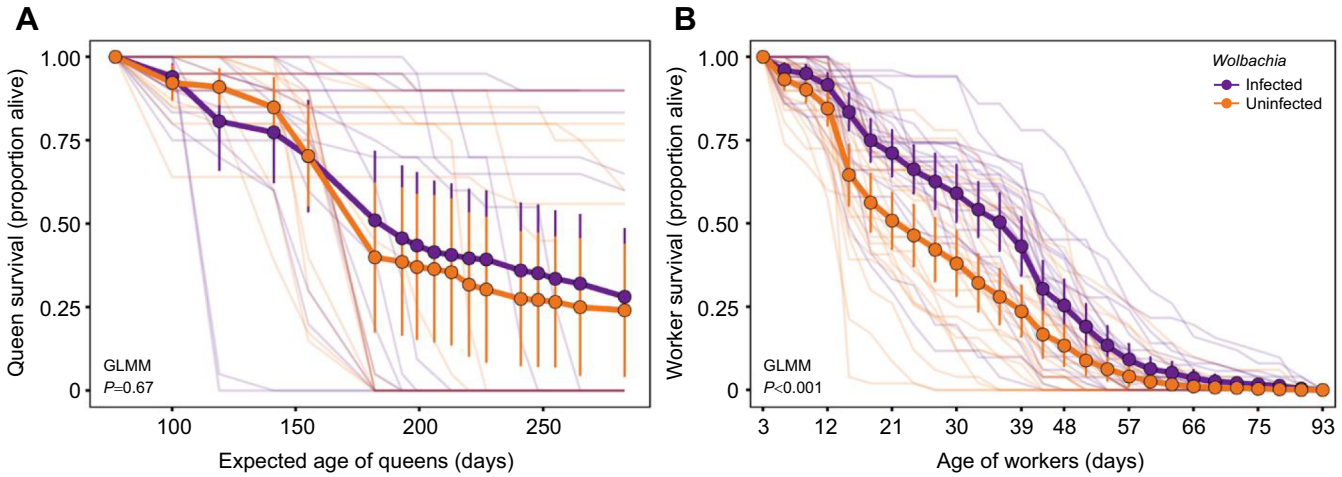

Fig. 2. Survival differences are dependent on *Wolbachia* infection and caste. (A) Within queens, infected and uninfected groups have similar proportions of alive queens over time. (B) Within workers, infected groups have higher proportions of alive workers over time than uninfected groups. The *x*-axis represents the estimated age of queens (A) or workers (B); the *y*-axis represents the proportion of alive queens (A) or workers (B). Dark lines represent the mean trend and lighter lines represent the trend of individual groups. The *P*-value estimate from the generalized linear mixed model (GLMM) is listed bottom left.

(GLMM: *z*-value=−2.98, *P*=0.002; Fig. 1A,B). Similarly, the per capita fecundity of *Wolbachia*-infected queens across 9 months was also higher when compared with that of uninfected queens (GLMER: *z*-value=−2.34, *P*=0.019; Fig. 1C,D).

Wolbachia-infected workers live longer than uninfected workers

Despite differences in egg laying of queens and in colony-level metabolic rates at some queen ages, *Wolbachia*-infected and uninfected queens had similar survival rates (GLMM, χ^2=0.2, *P*=0.067; Fig. 2A). Infected workers had higher survival (GLMM, χ^2=12, *P*<0.001; Fig. 2B) than uninfected workers.

Wolbachia-infected colonies have higher metabolic rates depending on the stage of the colony life cycle

Metabolic rate of whole colonies showed hypometric scaling with mass (Fig. 3E) and had a scaling coefficient of 0.58 (95% confidence interval, CI, 0.45–0.71), which is within the expected range (Fewell and Harrison, 2016; Shik et al., 2012). This means that the mass-specific metabolic rate will decrease with increasing mass of the ant colony. In contrast, τ, the scaling coefficient of metabolic rate (μW) of only the brood was 1.1 (95% CI 0.32–1.94), which suggests that as brood mass increases, mass-specific metabolic rate will increase similar to expectations by isometric scaling (Fig. 3F). Interestingly, for the groups of queens, mass-specific metabolic rate did not show a significant scaling effect with mass (Fig. 3G).

Wolbachia-infected pharaoh ant colonies with young queens (1–2 months old) had similar metabolic rates to the uninfected colonies (LM: *t*-value=0.75, d.f.=1, *P*>0.05; Fig. 3A). In contrast, *Wolbachia*-infected colonies with older queens (3 months old) had higher metabolic rates than uninfected colonies (LM: *t*-value=−3.95, d.f.=1, *P*=0.002; Fig. 3B).

We also compared the metabolic rates of different colony members when the colony was in early life cycle stages (1–2 month old queens). Metabolic rate of the brood (eggs to pupae) increased with the age of queens initially present in the colonies (LM: *t*-value=3.133, d.f.=1, *P*=0.006). Total brood mass also positively influenced brood metabolic rate (LM: *t*-value=2.68, d.f.=1, *P*=0.016), and total number of brood had no effect (LM: *t*-value=1.79, d.f.=1, *P*=0.091). The metabolic rates of groups of queens increased with the age of the queens (LM: *t*-value=3.51, d.f.=1, *P*<0.001) after statistically accounting for variation in mass of the queens. However, brood from these colonies did not show differences in metabolic rate when compared with uninfected brood (LM: *t*-value=0.58, d.f.=1, *P*>0.05; Fig. 3C). *Wolbachia*-infected groups of 15 queens that were 1–2 months old had similar metabolic rates to the uninfected queens (LM: *t*-value=0.86, d.f.=1, *P*=0.18; Fig. 3D) with no significant interaction of queen age with *Wolbachia* infection (LM: *t*-value=−0.99, d.f.=1, *P*>0.05).

DISCUSSION

We compared individual- and colony-level life history traits of infected and uninfected *M. pharaonis* colonies to elucidate the benefits and costs of *Wolbachia* infection. We found that *Wolbachia*-infected queens are more fecund, without a detectable metabolic cost. However, at a later colony life cycle stage (3 month old queens), when colonies peak in their productivity and reproductive investment (Singh and Linksvayer, 2020), infected colonies have higher metabolic rates. Despite increased fecundity by queens and higher colony-level metabolic rates, *Wolbachia* infection was not associated with decreased queen lifespan. Interestingly, in workers, which are obligately sterile, *Wolbachia* infection was associated with a longer lifespan. Thus, increased fecundity by queens and longer worker lifespans contribute to the higher growth rate and productivity that characterizes *Wolbachia*-infected colonies (Singh and Linksvayer, 2020).

Previous studies in solitary insects have shown that *Wolbachia* infection can cause increased stem cell differentiation or oogenesis [e.g. in *Drosophila mauritiana* (Fast et al., 2011) and *Asobara tabida* (Dedeine et al., 2001)], and similar effects on *M. pharaonis* queens might explain the effects on per capita fecundity that we observed in our study. Differences in the ability of infected workers to rear eggs might also contribute to the effects we observed. Cross-fostering infected queens with uninfected workers and vice versa will be useful to tease apart the role of queens, workers and queen–worker interaction on *Wolbachia*-induced phenotypes.

Given the increased fecundity by infected queens and increased growth of infected colonies (Singh and Linksvayer, 2020), we

Journal of Experimental Biology (2024) 227, jeb247168. doi:10.1242/jeb.247168

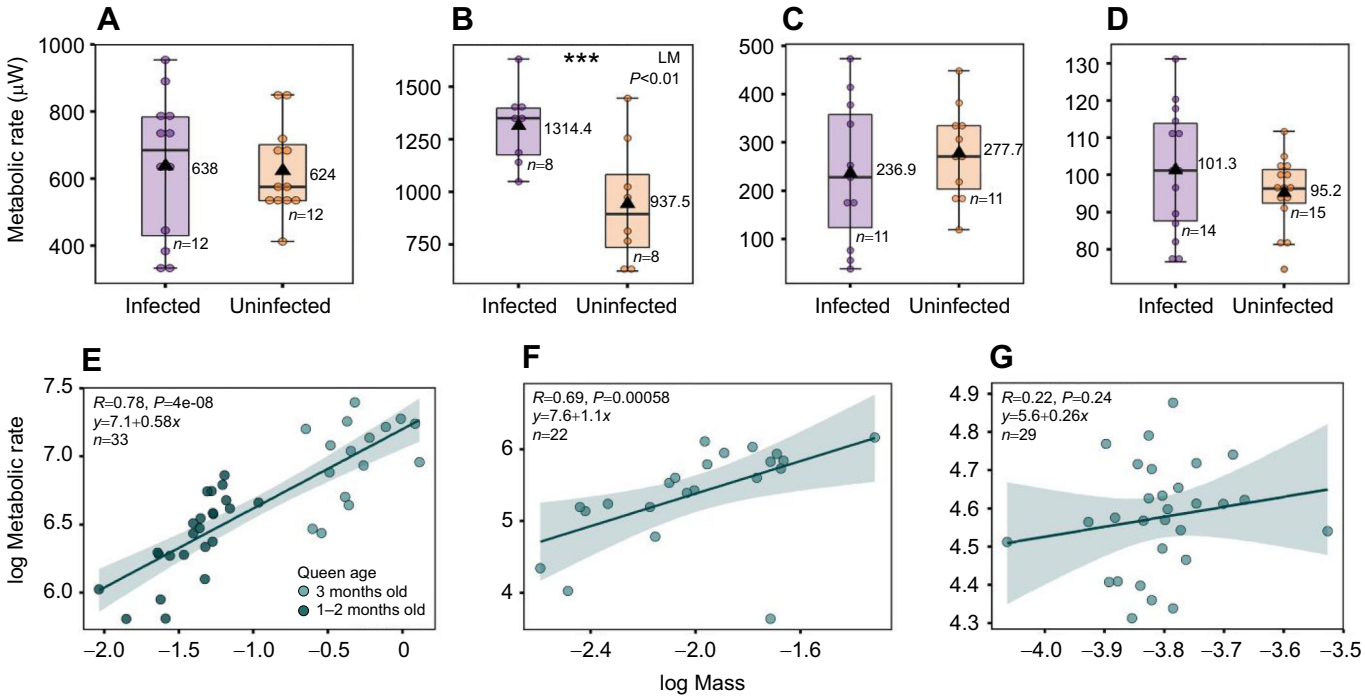

Fig. 3. Metabolic rates differ between infected and uninfected groups but are dependent on colony life cycle stage and colony component. (A) Infected and uninfected whole colonies with 1–2 month old queens have similar metabolic rates. (B) Infected colonies with 3 month old queens have higher metabolic rates than uninfected colonies. (C) Brood from infected and uninfected colonies with 1–2 month old queens have similar metabolic rates. (D) Groups of 15, 1–2 month old queens from infected and uninfected colonies have similar metabolic rates. The *x*-axis represents the *Wolbachia* infection status of the experimental group; the *y*-axis represents the metabolic rate of the groups. Box plots represent the quartile distribution of the raw data and the filled circles represent the individual raw values; the filled black triangle in the box plot represents the mean, which is also numerically listed besides the box plot. '*n*' represents the sample size. Asterisks indicate a significant difference between infected and uninfected groups, as determined by a linear model (***P<0.001). (E–G) A log–log plot of metabolic rate (μW) with mass (g) of whole colonies, with all our data combined (colonies with 1–3 month old queens; E), only the brood (colonies with 1–2 month old queens; F) and groups of approximately 15 queens (1–2 months old; G). '*R*' represents the Spearman rank correlation coefficient, '*P*' represents the significance of the correlation and '*n*' represents the sample size. The regression line equation is given top left in the form '*y*=*x*+*mc*', where '*m*' is the scaling coefficient.

expected that infected colonies would have higher metabolic rates. Furthermore, we expected that this energetic cost would be exacerbated by the maintenance cost of *Wolbachia* (Fleury et al., 2000; Fry et al., 2004; White et al., 2011). We did find this pattern in the 3 month old colonies. However, we did not find differences in metabolic rates of infected and uninfected whole colonies, brood and queens when the queens were young (1–2 months old). This suggests that *Wolbachia* infection does not have detectable energetic costs, or perhaps *Wolbachia* offsets any costs through a nutritional symbiosis, as found in the bed bug (*Cimex lectularius*; Hosokawa et al., 2010; Nikoh et al., 2014). Future studies comparing the metabolic rates of colonies and colony members across multiple colony life cycle stages will be helpful to better understand the energetic costs of *Wolbachia* infection.

A trade-off between fecundity and longevity has widely been observed within and between species, often assumed to be due to the costs of reproduction (Hammers et al., 2013; Kirkwood and Holliday, 1979). In contrast, social insects show a reversal in this fecundity–longevity tradeoff, where queens have high fecundity and long lifespans and workers are facultatively or obligately sterile and have short lifespans (Korb and Heinze, 2021). However, while we found an effect of *Wolbachia* infection on queen fecundity, we found no effect on queen lifespan. Surprisingly, we found that *Wolbachia*-infected workers, which are obligately sterile, did have longer lifespans. While both positive and negative effects of *Wolbachia* infection on the lifespan of solitary hosts have been observed (Fry et al., 2004; Min and Benzer, 1997; White et al., 2011), the mechanisms remain largely unknown. As workers are obligately sterile in *M. pharaonis* colonies, the presence of infected fecund queens that live as long as uninfected queens may be beneficial for *Wolbachia*, as more infected individuals can be produced over time. Future studies teasing apart the mechanisms by which *Wolbachia* infection causes higher egg-laying rates in *M. pharaonis* queens with no effect on lifespan, and longer lifespans in sterile *M. pharaonis* workers would provide more general insight into mechanisms linking reproduction, aging and metabolism.

Conclusion

Consistent with our previous results (Singh and Linksvayer, 2020), here we show that *Wolbachia* infection is beneficial for *M. pharaonis* ant colonies, at least in relatively benign laboratory conditions: infected young queens produced more eggs, infected colonies had higher metabolic rates during periods of peak productivity, and infected queens lived as long as uninfected queens, while infected workers outlived uninfected workers. These phenotypic effects of infection suggest that *Wolbachia* may have adapted to exploit the ant reproductive caste system without exacting a detectable cost on its ant host. The phenotypic and fitness consequences of *Wolbachia* infection that we observed, if also observed under more natural and in particular more stressful environmental conditions, are expected to be associated with the rapid spread of *Wolbachia* infection. However, the observation that *Wolbachia* infection is not universal in *M. pharaonis* colonies (Schmidt, 2010) implies that there may be certain environmental conditions under which the spread of *Wolbachia* within *M. pharaonis* populations is limited by potential

costs associated with the infection. Further experiments assessing the benefits and costs of *Wolbachia* infection under a variety of environmental conditions, especially stressful conditions, are needed to clarify these issues. We also note that future studies should clarify whether *M. pharaonis* populations harbor multiple *Wolbachia* strains and whether these strains differ in phenotypic effects on their hosts. More generally, further work is necessary to disentangle the precise molecular interactions between hosts and symbionts that produce the phenotypic effects we observed, which we inferred to be caused by *Wolbachia* infection.

Acknowledgements
We are grateful to Dr Juergen Liebig for letting us safely house the ants at Arizona State University, Xuaohui Guo in the Fewell lab and Trevor Fox in the Harrison lab at the Arizona State University for assisting with metabolic rate measurement, and the Linksvayer lab members for their feedback at various steps of the study.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: R.S., J.H.F., J.F.H., T.A.L.; Methodology: R.S., T.A.L., J.H.F., J.F.H.; Validation: R.S., J.H.F., J.F.H., T.A.L.; Formal analysis: R.S., T.A.L., S.S.; Investigation: R.S.; Resources: T.A.L., J.F.H., J.H.F.; Data curation: R.S., S.S.; Writing - original draft: R.S., T.A.L.; Writing - review & editing: R.S., T.A.L., S.S., J.H.F., J.F.H., T.A.L.; Visualization: R.S., T.A.L., S.S.; Supervision: T.A.L., J.F.H., J.H.F.; Project administration: T.A.L.; Funding acquisition: T.A.L.

Funding
This work was funded by the National Science Foundation (IOS-1452520) awarded to T.A.L.

Data availability
Data files and detailed R scripts are available from the Dryad digital repository (Suresh et al., 2023): https://doi.org/10.5061/dryad.0zpc8672b

References
Bates, D., Mächler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1-48. doi:10.18637/jss.v067.i01

Bolker, B. M., Brooks, M. E., Clark, C. J., Geange, S. W., Poulsen, J. R., Stevens, M. H. H. and White, J.-S. S. (2009). Generalized linear mixed models: a practical guide for ecology and evolution. *Trends Ecol. Evol.* **24**, 127-135. doi:10.1016/j.tree.2008.10.008

Bouwma, A. M., Ahrens, M. E., DeHeer, C. J. and DeWayne Shoemaker, D. (2006). Distribution and prevalence of *Wolbachia* in introduced populations of the fire ant *Solenopsis invicta*. *Insect Mol. Biol.* **15**, 89-93. doi:10.1111/j.1365-2583.2006.00614.x

de Bekker, C., Will, I., Das, B. and Adams, R. M. M. (2018). The ants (Hymenoptera: Formicidae) and their parasites: effects of parasitic manipulations and host responses on ant behavioral ecology. *Myrmecol. News* **28**, 1-2.

Dedeine, F., Vavre, F., Fleury, F., Loppin, B., Hochberg, M. E. and Bouletreau, M. (2001). Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. *Proc. Natl. Acad. Sci. USA* **98**, 6247-6252. doi:10.1073/pnas.101304298

Dussutour, A. and Simpson, S. J. (2008). Description of a simple synthetic diet for studying nutritional responses in ants. *Insectes Soc.* **55**, 329-333. doi:10.1007/s00040-008-1008-3

Engelstädter, J. and Hurst, G. D. D. (2009). the ecology and evolution of microbes that manipulate host reproduction. *Annu. Rev. Ecol. Evol. Syst.* **40**, 127-149. doi:10.1146/annurev.ecolsys.110308.120206

Fast, E. M., Toomey, M. E., Panaram, K., Desjardins, D., Kolaczyk, E. D. and Frydman, H. M. (2011). *Wolbachia* enhance *Drosophila* stem cell proliferation and target the germline stem cell niche. *Science* **334**, 990-992. doi:10.1126/science.1209609

Fewell, J. H. and Harrison, J. F. (2016). Scaling of work and energy use in social insect colonies. *Behav. Ecol. Sociobiol.* **70**, 1047-1061. doi:10.1007/s00265-016-2097-z

Fleury, F., Vavre, F., Ris, N., Fouillet, P. and Boulétreau, M. (2000). Physiological cost induced by the maternally-transmitted endosymbiont *Wolbachia* in the *Drosophila* parasitoid, *Leptopilina heterotoma*. *Parasitology* **121**, 493-500. doi:10.1017/S0031182099006599

Fox, J. and Weisberg, S. (2019). *An R Companion to Applied Regression*, 3rd edn. Thousand Oaks, CA: Sage.

Fry, A. J., Palmer, M. R. and Rand, D. M. (2004). Variable fitness effects of *Wolbachia* infection in *Drosophila melanogaster*. *Heredity* **93**, 379-389. doi:10.1038/sj.hdy.6800514

Gruntenko, N. E., Ilinsky, Y. Y., Adonyeva, N. V., Burdina, E. V., Bykov, R. A., Menshanov, P. N. and Rauschenbach, I. Y. (2017). Various *Wolbachia* genotypes differently influence host *Drosophila* dopamine metabolism and survival under heat stress conditions. *BMC Evol. Biol.* **17**, 252. doi:10.1186/s12862-017-1104-y

Gruntenko, N. E., Karpova, E. K., Adonyeva, N. V., Andreenkova, O. V., Burdina, E. V., Ilinsky, Y. Y., Bykov, R. A., Menshanov, P. N. and Rauschenbach, I. Y. (2019). *Drosophila* female fertility and juvenile hormone metabolism depends on the type of *Wolbachia* infection. *J. Exp. Biol.* **222**, jeb195347. doi:10.1242/jeb.195347

Hammers, M., Richardson, D. S., Burke, T. and Komdeur, J. (2013). The impact of reproductive investment and early-life environmental conditions on senescence: support for the disposable soma hypothesis. *J. Evol. Biol.* **26**, 1999-2007. doi:10.1111/jeb.12204

Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X.-Y. and Fukatsu, T. (2010). *Wolbachia* as a bacteriocyte-associated nutritional mutualist. *Proc. Natl. Acad. Sci. USA* **107**, 769-774. doi:10.1073/pnas.0911476107

Kautz, S., Rubin, B. E. R. and Moreau, C. S. (2013). Bacterial infections across the ants: frequency and prevalence of *Wolbachia, Spiroplasma*, and *Asaia*. *Psyche*. doi:10.1155/2013/936341

Kirkwood, T. B. and Holliday, R. (1979). The evolution of ageing and longevity. *Proc. R. Soc. Lond. B Biol. Sci.* **205**, 531-546. doi:10.1098/rspb.1979.0083

Korb, J. and Heinze, J. (2021). Ageing and sociality: why, when and how does sociality change ageing patterns? *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **376**, 20190727. doi:10.1098/rstb.2019.0727

Lee, C.-C., Lin, C.-Y., Tseng, S.-P., Matsuura, K. and Yang, C.-C. S. (2020). Ongoing coevolution of *Wolbachia* and a widespread invasive ant, *Anoplolepis gracilipes*. *Microorganisms* **8**, 1569. doi:10.3390/microorganisms8101569

Lighton, J. R. B. (2018). *Measuring Metabolic Rates: A Manual for Scientists*. Oxford University Press.

Lin, Y.-J., Yeh, C.-H., Wu, C.-Z. and Wu, L.-H. (2023). Climate and *Wolbachia* impacts on *Anoplolepis gracilipes* (Hymenoptera: Formicidae). *Biology* **12**, 1482. doi:10.3390/biology12121482

Min, K. T. and Benzer, S. (1997). *Wolbachia*, normally a symbiont of *Drosophila*, can be virulent, causing degeneration and early death. *Proc. Natl. Acad. Sci. USA* **94**, 10792-10796. doi:10.1073/pnas.94.20.10792

Moreau, C. S. (2020). Symbioses among ants and microbes. *Curr. Opin. Insect Sci.* **39**, 1-5. doi:10.1016/j.cois.2020.01.002

Mouton, L., Henri, H., Charif, D., Boulétreau, M. and Vavre, F. (2007). Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. *Biol. Lett.* **3**, 210-213. doi:10.1098/rsbl.2006.0590

Nikoh, N., Hosokawa, T., Moriyama, M., Oshima, K., Hattori, M. and Fukatsu, T. (2014). Evolutionary origin of insect-*Wolbachia* nutritional mutualism. *Proc. Natl. Acad. Sci. USA* **111**, 10257-10262. doi:10.1073/pnas.1409284111

Peacock, A. D., Sudd, J. H. and Baxter, A. T. (1955). Studies in pharaoh's ant, *Monomorium pharaonis* (L.). 11. Colony foundation. *Entomol. Mon. Mag.* **91**, 125-133.

Pontieri, L., Schmidt, A. M., Singh, R., Pedersen, J. S. and Linksvayer, T. A. (2017). Artificial selection on ant female caste ratio uncovers a link between female-biased sex ratios and infection by *Wolbachia* endosymbionts. *J. Evol. Biol.* **30**, 225-234. doi:10.1111/jeb.13012

Ramalho, M. O., Kim, Z., Wang, S. and Moreau, C. S. (2021). *Wolbachia* across social insects: patterns and implications. *Ann. Entomol. Soc. Am.* **114**, 206-218. doi:10.1093/aesa/saaa053

Reuter, M., Pedersen, J. S. and Keller, L. (2005). Loss of *Wolbachia* infection during colonisation in the invasive Argentine ant, *Linepithema humile*. *Heredity* **94**, 364-369. doi:10.1038/sj.hdy.6800601

Rey, O., Estoup, A., Facon, B., Loiseau, A., Aebi, A., Duron, O., Vavre, F. and Foucaud, J. (2013). Distribution of endosymbiotic reproductive manipulators reflects invasion process and not reproductive system polymorphism in the little fire ant *Wasmannia auropunctata*. *PLoS One* **8**, e58467. doi:10.1371/journal.pone.0058467

Reynolds, K. T., Thomson, L. J. and Hoffmann, A. A. (2003). The effects of host age, host nuclear background and temperature on phenotypic effects of the virulent *Wolbachia* strain popcorn in *Drosophila melanogaster*. *Genetics* **164**, 1027-1034. doi:10.1093/genetics/164.3.1027

Russell, J. A. (2012). The ants (Hymenoptera: Formicidae) are unique and enigmatic hosts of prevalent *Wolbachia* (Alphaproteobacteria) symbionts. *Myrmecol. News* **16**, 7-23.

Russell, J. A., Goldman-Huertas, B., Moreau, C. S., Baldo, L., Stahlhut, J. K., Werren, J. H. and Pierce, N. E. (2009). Specialization and geographic isolation among *Wolbachia* symbionts from ants and lycaenid butterflies. *Evolution* **63**, 624-640. doi:10.1111/j.1558-5646.2008.00579.x

Schmidt, A. M. (2010). *The Invasion Biology and Sociogenetics of Pharaoh Ants*. Museum Tusculanum.

Shik, J. Z., Hou, C., Kay, A., Kaspari, M. and Gillooly, J. F. (2012). Towards a general life-history model of the superorganism: predicting the survival, growth

and reproduction of ant societies. *Biol. Lett.* **8**, 1059-1062. doi:10.1098/rsbl.2012.0463

Shoemaker, D. D., Katju, V. and Jaenike, J. (1999). Wolbachia and the evolution of reproductive isolation between *Drosophila recens* and *Drosophila subquinaria*. *Evolution* **53**, 1157-1164. doi:10.2307/2640819

Shoemaker, D. D., Ross, K. G., Keller, L., Vargo, E. L. and Werren, J. H. (2000). *Wolbachia* infections in native and introduced populations of fire ants (*Solenopsis* spp.). *Insect Mol. Biol.* **9**, 661-673. doi:10.1046/j.1365-2583.2000.00233.x

Singh, R. and Linksvayer, T. A. (2020). Wolbachia-infected ant colonies have increased reproductive investment and an accelerated life cycle. *J. Exp. Biol.* **223**, jeb220079. doi:10.1242/jeb.220079

Suresh, S. et al. (2023). Wolbachia-infected pharaoh ant colonies have higher egg production, metabolic rate, and worker survival [Dataset]. Dryad. https://doi.org/10.5061/dryad.0zpc8672b

Treanor, D. and Hughes, W. O. H. (2019). Limited female dispersal predicts the incidence of *Wolbachia* across ants (Hymenoptera: Formicidae). *J. Evol. Biol.* **32**, 1163-1170. doi:10.1111/jeb.13510

Tseng, S.-P., Wetterer, J. K., Suarez, A. V., Lee, C.-Y., Yoshimura, T., Shoemaker, D. and Yang, C.-C. S. (2019). Genetic diversity and *Wolbachia* infection patterns in a globally distributed invasive ant. *Front. Genet.* **10**, 838. doi:10.3389/fgene.2019.00838

Tsutsui, N. D., Kauppinen, S. N., Oyafuso, A. F. and Grosberg, R. K. (2003). The distribution and evolutionary history of *Wolbachia* infection in native and introduced populations of the invasive Argentine ant (*Linepithema humile*): Phylogeography of *Wolbachia* in Argentine ants. *Mol. Ecol.* **12**, 3057-3068. doi:10.1046/j.1365-294X.2003.01979.x

Van Borm, S., Wenseleers, T., Billen, J. and Boomsma, J. J. (2001). *Wolbachia* in leafcutter ants: a widespread symbiont that may induce male killing or incompatible matings. *J. Evol. Biol.* **14**, 805-814. doi:10.1046/j.1420-9101.2001.00321.x

Waters, J. S., Holbrook, C. T., Fewell, J. H. and Harrison, J. F. (2010). Allometric scaling of metabolism, growth, and activity in whole colonies of the seed-harvester ant *Pogonomyrmex californicus*. *Am. Nat.* **176**, 501-510. doi:10.1086/656266

Wenseleers, T., Ito, F., Van Borm, S., Huybrechts, R., Volckaert, F. and Billen, J. (1998). Widespread occurrence of the micro-organism *Wolbachia* in ants. *Proc. Biol. Sci.* **265**, 1447-1452. doi:10.1098/rspb.1998.0456

Wenseleers, T., Sundström, L. and Billen, J. (2002). Deleterious *Wolbachia* in the ant *Formica truncorum*. *Proc. Biol. Sci.* **269**, 623-629. doi:10.1098/rspb.2001.1927

Werren, J. H., Baldo, L. and Clark, M. E. (2008). Wolbachia: master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741-751. doi:10.1038/nrmicro1969

White, J. A., Kelly, S. E., Cockburn, S. N., Perlman, S. J. and Hunter, M. S. (2011). Endosymbiont costs and benefits in a parasitoid infected with both *Wolbachia* and *Cardinium*. *Heredity* **106**, 585-591. doi:10.1038/hdy.2010.89

Zélé, F., Altıntaş, M., Santos, I., Cakmak, I. and Magalhães, S. (2020). Population-specific effect of Wolbachia on the cost of fungal infection in spider mites. *Ecol. Evol.* **10**, 3868-3880. doi:10.1002/ece3.6015

Zug, R. and Hammerstein, P. (2015). Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biol. Rev.* **90**, 89-111. doi:10.1111/brv.12098

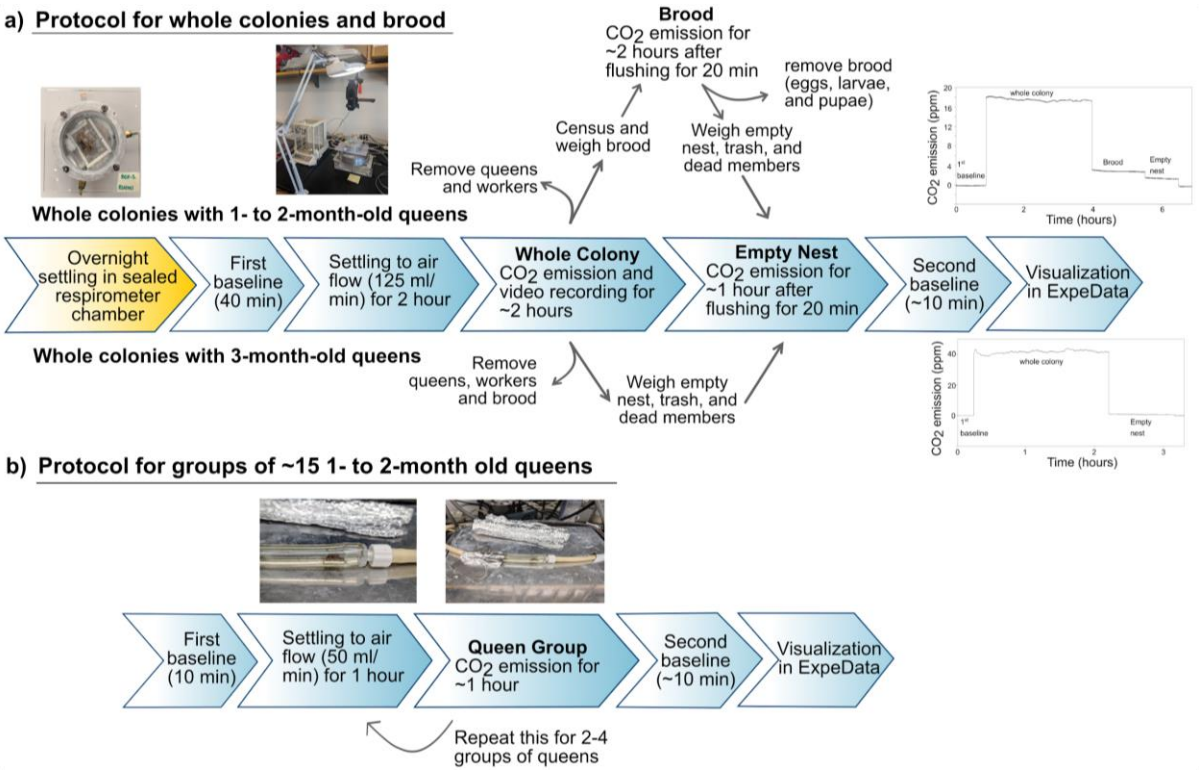

Fig. S1. Setup used for estimating metabolic rates. (a) Detailed steps for measuring the CO_2 emission from whole colonies and brood with 1- to 2-month-old queens (top half) and whole colonies with 3-month-old queens (bottom half). (b) Detailed steps to measure CO_2 emission from groups of 1- to 2-month-old queens. Yellow color highlights the steps done a day prior to the measurement, whereas the blue color highlights the steps performed on the day of recording CO_2 emission on a respirometer.

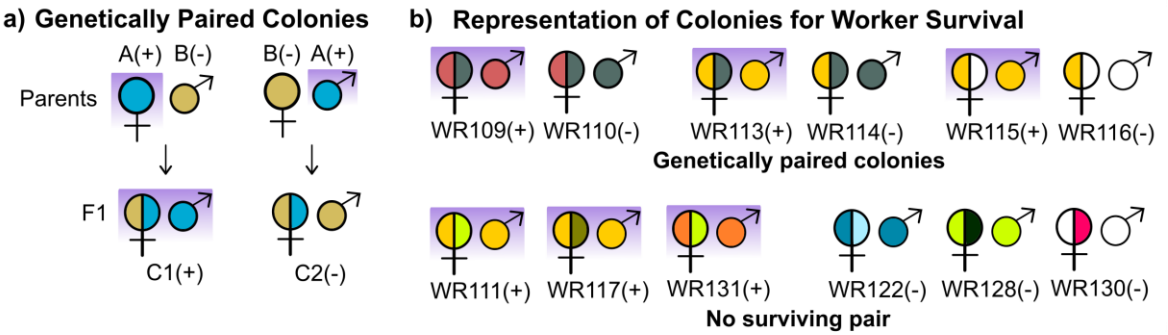

Fig. S2. Reciprocal crossing scheme to produce genetically paired *Monomorium pharaonis* colonies that differ in *Wolbachia* infection for comparing worker survival.

(a) We used a reciprocal crossing scheme to control for genotype when comparing *Wolbachia*-driven differences in life history traits of colonies and colony members. 'A' and 'B' represent sample parent colony ID of differing genotypes and 'C1' and 'C2' represent sample F1 colony ID. (b) A graphical representation of genetic diversity of the colonies used for comparing worker survival. We used 3 pairs of colonies that were expected to be genetically similar but have different *Wolbachia* infection status (top half). We also used colonies that did not have a surviving counterpart (bottom half). Each color represents a unique colony ID from heterogeneous stock colonies used for setting up reciprocal crosses. '(+)' following the colony ID and with a purple background means that the colony is infected with *Wolbachia*. '(-)' with a white background means that the colony is uninfected.

Supplementary Materials and Methods

Comparing metabolic rate differences

We performed respirometry measurements of whole colonies and brood in Dr. Jennifer Fewell's Lab and respirometry measurements of groups of queens in Dr. Jon Harrison's lab at Arizona State University (Tempe, AZ) by transporting experimental *Monomorium pharaonis* colonies in 50 ml falcon tubes in a cabin bag on a flight. These colonies were stored in Dr. Juergen Leibig's ant room chamber at Arizona State University (Tempe, AZ) at 26^{o}C with ± 60% relative humidity. The colonies were fed *ad libitum* synthetic agar along with dried mealworms twice a week. We transported the colonies twice, once in February 2018 when the colonies had 3-month-old queens and then again in November 2019 when the colonies had 1-month-old queens.

A day before recording CO_2 emissions from whole colonies, we censused and weighed the whole colony and then kept them overnight in a sealed respirometer chamber to settle in the new space (Fig. S1). The inner wall of the respirometer chamber was coated with a layer of fluon and the internal openings of the chamber were covered by a fine mesh to prevent ant escapes. For colonies with young queens (<2-month-old), since we wanted to record CO_2 emissions from the brood after recording from an experimental colony, we also transferred a small water tube into the respirometer chamber for this overnight settling and CO_2 emission recording the next day to alleviate any possible stress due to long periods of absence of humidity (Fig S1a). We started the CO_2 emission recording by first measuring a 40-min long first baseline on LiCor-7000, which served as a reference for any differences in CO_2 levels between the reference and sample cells of LiCor-7000 (Fig S1a). Following this we connected the respirometer chamber with our experimental colonies to the LiCor-7000 setup and allowed the colonies to settle for another 2 hours at a flow rate of 125 ml/min. After this we started recording the CO_2 emission from the respirometer chamber, ambient temperature, and the humidity within the respirometer chamber for approximately two hours till the colonies showed a stable emission. Post this, the colonies were taken out from the

respirometer chamber and we used two different approaches between the colonies with queens younger than 2 months and colonies with 3-month-old queens. For colonies with 3-month-old queens, we returned the whole colony back to its original box, and weighed the empty nest, with any possible trash and dead colony members before placing the empty nest back to the respirometer chamber (Fig S1a). For colonies with queens younger than 2 months, we moved all the queens and workers from the colony back to its original box, censused and weighed the brood (eggs, larvae, pre-pupae, and pupae) and then proceeded to record CO_2 emission from just the brood for approximately two hours in the same way as described above (Fig S1a). After recording from the brood, we returned all the brood back to its original colony box but retained any dead colony members and trash within the respirometer chamber and nest. For both our approaches, we weighed the empty nest with dead colony members and trash and then sealed them in the respirometer chamber. We then passed CO_2-free dry air at a flow rate of 125 ml/min for 20 min to flush any environmental CO_2 that made its way into the chamber during the entire process (Fig S1a). We recorded CO_2 emissions from the empty nest for approximately an hour as a reference for background CO_2 emissions (Fig S1a). After this we removed the respirometer chamber, connected the tubings to each other and recorded the second baseline for 10 min to account for any drift over the course of recording. We also recorded videos of the whole colonies over the course of CO_2 emission measurements to later compare activity level differences.

We also estimated metabolic rates of groups of approximately 15 young queens in 2019 by storing them in tubing with fine mesh on both ends to prevent ant escapes (Fig S1b). We recorded CO_2 emission rate from these groups of queens using the differential mode in LiCor-6252 gas analyzer by flowing dry CO_2-free air at 50 ml/min through mass flow controllers (50 ml/min max, set to 100%). The queens were collected from replicate experimental colonies which had already been measured and the queens were weighed before recording their CO_2 emission. We first recorded a 10-min long first baseline. Following this, we connected the tube with the group of queens, allowed the group to settle

to the airflow for about an hour and then started recording CO_2 emission from this group for another hour before returning them back to their respective colonies (Fig S1b). After this we added another group of queens to the same tube, following the exact same protocol. We measured one to four groups of queens per day while alternating between infected and uninfected groups (Fig S1b). Once we were done recording CO_2 emissions from the groups of queens for the day, we recorded a 10-min long second baseline.

The data was initially visualized and analyzed using the ExpeData software (release 1.9.13) from Sable Systems International to obtain mean values of CO_2 emission, humidity, and temperature over a period of stable CO_2 emission per experimental group. We corrected for shift between the first and secondbaseline values using ExpeData software before exporting the data as a CSV file for further analysis in R.We subtracted mean values of CO_2 emission of empty nests from that of the whole colonies and the brood to remove the background noise.

We compared the activity levels of whole colonies using Swarm Sight Motion Analysis software [50]. The activity data was then compiled in a CSV file to generate mean values per colony and for further analysis in R.

Creating genetically paired colonies with and without Wolbachia *for the worker lifespan assay*

We used six *Wolbachia*-infected and six uninfected heterogeneous stock colonies to set up reciprocal crosses (Fig S2). For reciprocal crossing, we artificially induced the production of new queens and males in six heterogenous stock colonies of known pedigree per infection status. Four weeks post this, we collected as many darkly pigmented queen and male pupae per colony as possible and stored them separately in petri dishes along with 50 workers of the same genotype. To produce genetically paired colonies, we set up a cross between 15 *Wolbachia*-infected virgin queens of genotype A and 10 uninfected virgin males

of genotype B and another cross between 15 uninfected virgin queens of genotype B and 10 *Wolbachia*-infected males of genotype A (Fig. S3). Such a cross produced pairs of colonies that are genetically very similar to each other but only one of them is infected with *Wolbachia* since *Wolbachia* is maternally inherited.

Verification of Wolbachia *infection status*

We tested for the presence of *Wolbachia* in 5-8 workers collected from outside the nest of each initial stock colony and each replicate study colony. We detected *Wolbachia* infection by PCR-amplification of fragments from two *Wolbachia* genes: *ftsZ*, a cell division protein-encoding gene, and *wsp,* a cell surface protein-encoding gene; and we further confirmed the presence of bacterial DNA by amplifying a fragment of the bacterial *16S rRNA* gene (see Pontieri et al. 2017; Singh and Linksvayer 2020 for primers and additional details). We used positive and negative controls for each PCR run. As a positive control, we used DNA from an individual known to carry *Wolbachia*, whereas as a negative control we replaced the genomic DNA with deionized water. The researcher who conducted the screening was blind with respect to the pedigree information and the inferred infection status of the colony from where we collected the worker samples.