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Artificial selection on ant female caste ratio uncovers a link between female-biased sex ratios and infection by *Wolbachia* endosymbionts

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Abstract

Social insect sex and caste ratios are well-studied targets of evolutionary conflicts, but the heritable factors affecting these traits remain unknown. To elucidate these factors, we carried out a short-term artificial selection study on female caste ratio in the ant *Monomorium pharaonis*. Across three generations of bidirectional selection, we observed no response for caste ratio, but sex ratios rapidly became more female-biased in the two replicate high selection lines and less female-biased in the two replicate low selection lines. We hypothesized that this rapid divergence for sex ratio was caused by changes in the frequency of infection by the heritable bacterial endosymbiont *Wolbachia*, because the initial breeding stock varied for *Wolbachia* infection, and *Wolbachia* is known to cause female-biased sex ratios in other insects. Consistent with this hypothesis, the proportions of *Wolbachia*-infected colonies in the selection lines changed rapidly, mirroring the sex ratio changes. Moreover, the estimated effect of *Wolbachia* on sex ratio (~13% female bias) was similar in colonies before and during artificial selection, indicating that this *Wolbachia* effect is likely independent of the effects of artificial selection on other heritable factors. Our study provides evidence for the first case of endosymbiont sex ratio manipulation in a social insect.

Introduction

Social insect colonies have long served as models for evolutionary conflicts of interest over resource investment into reproductives of different sex (i.e. queens vs. males; sex ratio) and investment into females of different caste (i.e. queens vs. sterile workers; caste ratio. Trivers & Hare, 1976; Ratnieks *et al.*, 2006; Meunier *et al.*, 2008). However, for potential conflicts over sex ratio or caste ratio to actually be realized in terms of evolutionary responses to selection, there must be heritable variation for caste ratio and sex ratio. Although the molecular, epigenetic and endocrine underpinnings of caste development have been the focus of intensive study (Corona *et al.*, 2016), the genetic basis of variation for social insect caste ratio and sex ratio has remained relatively little studied (Linksvayer, 2006; Anderson *et al.*, 2008; Schwander *et al.*, 2010).

Caste has long been considered to be an exemplar polyphenism, with alternate caste fates being dependent only on environmental conditions (i.e. 'environmental caste determination'), such as larval nutrition (Wheeler, 1986). However, for ants, a number of cases have been documented where larval caste fate is strongly influenced by larval genotype (i.e. 'genetic caste determination', see Anderson *et al.*, 2008; Schwander *et al.*, 2010). In other species, larval caste fate has been found to be weakly influenced by larval genotype (Anderson *et al.*, 2008; Schwander *et al.*, 2010) or the genotypes of the mother queen or nursing workers (see Linksvayer, 2006; Anderson *et al.*, 2008). Although these genetic influences have typically been considered to be rare exceptions to the rule of strict environmental caste determination, in theory, genetic influences on

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caste should actually be ubiquitous (Anderson *et al.*, 2008; Schwander *et al.*, 2010). If there is conflict over caste fate, segregating queen-biasing alleles could represent a selfish strategy (i.e. 'royal cheats', see Hughes & Boomsma, 2008) and be maintained at an intermediate frequency by the balance of within- and between-colony selection (Anderson *et al.*, 2008; Van Dyken *et al.*, 2011). Alternatively, if queen-biasing alleles have overall deleterious fitness effects, they will still be maintained at low frequency by mutation–selection balance (Van Dyken *et al.*, 2011). In both cases, heritability for caste fate is expected to be greater than zero. Similarly, heritable variation for sex ratio is also expected to be maintained within populations (Linksvayer, 2006; Van Dyken *et al.*, 2011).

Besides genetic influences on caste ratio and sex ratio, it is also conceivable that other heritable factors, such as vertically transmitted endosymbionts, bias caste or sex allocation, potentially leading to another type of evolutionary conflict, between hosts and endosymbionts (Cordaux *et al.*, 2011). The bacterial endosymbiont *Wolbachia* is a particularly well-known manipulator of sex ratio in arthropods (Moran *et al.*, 2008; Werren *et al.*, 2008). Being exclusively maternally inherited, *Wolbachia* spreads by increasing host reproduction through females at the expense of reproduction through males, using a variety of mechanisms (reviewed in Werren *et al.*, 2008). Female-biasing effects of *Wolbachia* on colony sex ratio have been predicted (Chapuisat & Keller, 1999), and such expectations extend to effects on caste ratio when workers are sterile (or produce only males) and thus represent a dead end for *Wolbachia* transmission. However, despite its widespread occurrence in ants (Russell, 2012), no study has yet documented the phenotypic effects of *Wolbachia* on ants (Andersen *et al.*, 2012; Russell, 2012).

Artificial selection provides a powerful approach to experimentally study the genetic basis of phenotypic variation, including the most basic question of whether phenotypes are heritable and can respond to short-term selection (Fuller *et al.*, 2005). Honeybee researchers have successfully used artificial selection (paired with artificial insemination) to study the genetic basis of honeybee traits such as pollen hoarding (Page & Fondrk, 1995). Unfortunately, the life history of most other social insects, including most ant species, precludes controlled breeding, so that artificial selection on ant caste ratio (or any other ant trait) has to our knowledge never been attempted.

To experimentally verify whether caste ratio is heritable and can respond to selection, we performed an artificial selection study on caste ratio in the pharaoh ant *Monomorium pharaonis*. This ant species has the following attributes that make it especially well suited for such studies: (i) there are no mating flights and controlled crosses can readily be made in the laboratory; (ii) generation time is short relative to most other ants; (iii) hundreds of colonies are readily maintained in the laboratory; (iv) workers lack ovaries so that worker-produced males do not complicate breeding programmes; and (v) colonies can be experimentally induced to produce new queens and males by removing existing queens (Schmidt *et al.*, 2011a). Furthermore, because some, but not all, of the colonies in our initial breeding population were infected by *Wolbachia* (Schmidt *et al.*, 2010), we were able to investigate potential effects of *Wolbachia* on colony caste ratio and sex ratio.

Material and methods

Creation of the initial breeding stock for artificial selection

Artificial selection experiments are usually initiated with a highly genetically variable base population because genetic variation for the selected trait is required for a response to selection (Fuller *et al.*, 2005). In 2008, we created a genetically variable *M. pharaonis* breeding stock by systematically intercrossing eight inbred and genetically distinct laboratory lineages for two generations. These eight lineages were derived from *M. pharaonis* samples spanning the global species range (Schmidt *et al.*, 2010. See Tables S1 and S2 in Appendix S1, for information about the eight lineages; see Fig. S1 in Appendix S1 for the crossing scheme). These crosses resulted in 158 genetically diverse *M. pharaonis* colonies (hereafter 'Generation 0', Table S4 in Appendix S1), each of which was expected to contain alleles from three of eight of the founding inbred lineages (Fig. S1 in Appendix S1).

Each new colony was started with 15 mated queens (*M. pharaonis* is naturally highly polygynous, i.e. with multiple queens per colony), as well as eggs, larvae of several stages and workers supplemented from donor colonies, as pharaoh ant queens are unable to start colonies without existing brood and workers (Peacock *et al.*, 1955). We inspected the colonies every 10 days over a 3-month period for sexual larvae production, and, when found, we removed all such larvae. As development time from egg to adult is approximately 28 days (Schmidt *et al.*, 2011b), this procedure over 3 months ensured that all the brood subsequently surveyed and used to breed and create subsequent generations were offspring of the original 15 queens, and not derived from brood supplemented from donor colonies (Fig. S1 in Appendix S1).

Bidirectional artificial selection on colony caste ratio

In May 2009, we dequeened the colonies to trigger sexual production (Schmidt *et al.*, 2011b). After 4 weeks, we surveyed colonies every 5 days and counted and

removed worker, gyne (i.e. virgin queens) and male pupae at each survey until no brood was left. Gyne and male pupae from each colony were allowed to mature into adults in separate fluon-coated Petri dishes with adult workers, after which they were used as parents for the next generation.

After the last survey, we calculated the caste ratio for each colony as the total number of gyne pupae produced divided by the total number of female pupae produced (worker plus gynes). We weighted these caste ratio estimates by the log10 of total colony productivity (i.e. the total number of worker, gyne and male pupae produced), because we had more confidence in caste ratio estimates for larger, more productive colonies. We ranked colonies according to these weighted values (i.e. log10(productivity)*(caste ratio)) and selected the 24 highest and the 24 lowest ranked colonies (each initiated with 15 mated queens), which in turn were randomly split into two high and two low artificial selection replicate lines, respectively. The two high replicates (hereafter 'High1' and 'High2') each comprised 12 high caste ratio value colonies, whereas the two low replicates (hereafter 'Low1' and 'Low2') each comprised 12 low caste ratio value colonies. Note that to be selected in this and subsequent generations, colonies had to produce enough males or gynes to contribute to the creation of the next generation, which meant that the caste ratio of the low lines was constrained (e.g. colonies that produced only workers could not be selected). Selected colonies at the end of each generation are hereafter referred to as 'selected parents'. In July 2009, gyne and male offspring from the selected parents within each replicate line were mated randomly to found a new set of colonies, hereafter 'Generation 1' (Table S4 in Appendix S1).

To create and survey two more generations of colonies, hereafter named 'Generation 2' and 'Generation 3' (Table S4 in Appendix S1), colonies consisting of newly mated gynes (i.e. offspring of selected parents) were (i) supplied with workers and brood from donor colonies; (ii) screened for sexual larvae production over a 3-month period to remove any sexual larvae that could have been introduced from donor colonies; (iii) dequeened to induce the production of new sexuals; (iv) surveyed for worker, gyne and male pupae production; and (v) ranked according to their weighted caste ratio values, to select the 24 highest and the 24 lowest caste ratio colonies to parent the subsequent generation. Information about date of creation, number of colonies in each replicate line and number of gynes and males used to initiate the colonies for every generation can be found in Table S3 in Appendix S1.

All colonies used in the artificial selection experiment were kept in climate-controlled rooms at 27 ± 1 °C, approximately 50% RH, and a 12:12-h light/dark cycle. Colonies were supplied *ad libitum* with water tubes plugged with cotton wool and a diet of boiled liver, boiled egg yolk and *Tenebrio molitor* larvae.

Inferred *Wolbachia* infection status of colonies and experimental validation

Two of the eight original lineages used to establish our initial breeding stock were infected by the bacterial endosymbiont *Wolbachia* (Schmidt *et al.*, 2010; see Table S1 in Appendix S1). Because *Wolbachia* is expected to be faithfully maternally inherited, we assumed that the *Wolbachia* infection status of a given colony could be accurately predicted by the infection status of its original maternal lineage (see Fig. S1 in Appendix S1 for an example). Based on this assumption, we determined the expected infection status of all colonies in our selection experiment by reconstructing their pedigree history (see Table S4 in Appendix S1) and labelled colonies as 'infected' or 'uninfected' based on their inferred *Wolbachia* infection status. During all steps of the study, we were blind with respect to the pedigree history and *Wolbachia* infection status of each colony.

To validate our inferences about infection status, we screened three queens from each of 66 colonies (at least 20 per generation) for the presence of *Wolbachia* (see Table S5 in Appendix S1 for details), using a PCR-based assay based on specific amplification of the *Wolbachia* genes *Wsp* and *ftsZ* (see '*Wolbachia* detection protocol' in Appendix S1). We scored a colony as *Wolbachia*-infected when all three queens screened positive and noninfected when all three queens were negative. Of 66 screened colonies, 95% (63 of 66) matched the expected infection status based on original maternal lineage infection status. Of the three colonies that did not match our predictions, two scored negative for the infection while expected to be infected, and one scored positive for the infection while expected to be uninfected.

Statistical analysis

We performed all statistical analyses in R v. 3.1.2 (R Core Team, 2014). Data set and R scripts used for analyses and figures are provided in Appendices S2 and S3. Unless otherwise specified, all caste ratio analyses were conducted only on colonies that produced at least one female pupa, whereas all sex ratio analyses (defined as the ratio of gyne pupae to the total number of sexual pupae produced) were performed only on colonies that produced at least one sexual pupa (i.e. a gyne or a male).

First, we verified that the within-generation artificial selection we imposed was successful by confirming that, in each generation, selected parents in the high selection replicate lines had higher caste ratio values than those in the low replicate lines. For each generation,

we ran a generalized linear model (GLM) with a logit-link function and quasibinomial error structure to account for overdispersion of the response variable, and with caste ratio as response variable and replicate selection lines ('High1', 'High2', 'Low1' and 'Low2') and log10-transformed colony total productivity as fixed categorical predictors. We performed planned comparisons (one-tailed tests) with false discovery rate (FDR) correction, under the hypotheses that each high replicate line displayed a higher caste ratio value than both low replicate lines using the R package multcomp (Hothorn *et al.*, 2008).

Next, we investigated whether there was a response to selection for caste ratio in the high and low lines by running a GLM with a logit-link function and quasibinomial error structure for all the colonies in the three generations of selection. The model included caste ratio as response variable; selection lines ('high' and 'low'), generations (1, 2 and 3) and their second-order interaction as fixed categorical predictors; replicate lines ('High1', 'High2', 'Low1' and 'Low2') as fixed factor nested within selection line; and log10-transformed colony total productivity as a continuous predictor. We assessed the significance of each term using a sequential analysis of deviance. To investigate whether the high and low selection lines differed for sex ratio, we ran a similar GLM described above, except with sex ratio replacing caste ratio as the response variable, for all the colonies in the three generations of selection.

We also investigated whether the proportion of *Wolbachia*-infected colonies changed with selection. First, we used an exact binomial test to assess whether the proportions of *Wolbachia*-infected and uninfected colonies differed in the original breeding population. We then used Fisher's exact tests to investigate whether, in each generation of selection, the proportion of *Wolbachia*-infected colonies differed across replicate lines.

Because the proportion of *Wolbachia*-infected colonies in each replicate line appeared to be positively correlated to colony sex ratio (see Results section), we hypothesized that *Wolbachia* infection (or another heritable factor strongly correlated with *Wolbachia*) caused female-biased sex ratios in *M. pharaonis*. Alternatively, sex ratio could be affected by factors independent of *Wolbachia* that responded to our selection regime for caste ratio. To disentangle the effects of *Wolbachia* and selection on sex ratio, (i) we used two separate GLMs, with quasibinomial error structure and logit-link function on a pooled data set consisting of all the colonies in the three selection generations to estimate the separate effects of each factor. The first model included selection lines ('high' and 'low') and log10-transformed colony total productivity as predictors, whereas the second model included *Wolbachia* infection status ('infected' or 'uninfected') and log10-transformed colony total productivity as predictors. (ii) We estimated the effect of *Wolbachia* on sex ratio before and after the onset of artificial selection. We reasoned that if the effect of *Wolbachia* on sex ratio was confounded with the effects of artificial selection on sex ratio (e.g. due to changes in ant genotypic frequencies), we would not observe an association between *Wolbachia* and sex ratio *before* the onset of artificial selection. Specifically, we used a similar GLM as above, with *Wolbachia* infection status and log10-transformed colony total productivity as predictors, on all the colonies in the initial breeding population.

We also performed an intraline comparison for sex ratios between infected and uninfected colonies to further confirm that *Wolbachia* infection was associated with the strong sex ratio difference observed between high and low selection lines (see Results). Specifically, we ran a GLM with a logit-link function and a quasibinomial error structure for each of the two selection lines. Each model included sex ratio as response variable, the *Wolbachia* infection status of the colony as a fixed categorical predictor and log10-transformed colony total productivity as a continuous predictor.

To assess whether the association between female-biased sex ratios and *Wolbachia* infection status of the colony we observed in the selection lines (see Results) was caused by differences in gyne or male production between infected and uninfected colonies, we investigated whether infected and uninfected colonies in the selection lines differed in terms of total number of gynes and males produced. Specifically, we ran a GLM with a log-link function and a quasi-Poisson error structure for each sex on a pooled data set consisting of all colonies in the selection lines that produced at least one sexual pupa. Each model included the total number of males or gynes produced as response variable and the *Wolbachia* infection status of the colony as a fixed categorical predictor.

To further elucidate the putative mechanism through which *Wolbachia* might cause female-biased sex ratios in *M. pharaonis*, we investigated whether infected and uninfected colonies in the whole experiment differed in terms of gyne, male and worker production, as well as total colony productivity, caste ratio, sex ratio and female-to-male ratio. We ran GLMs with a log-link function and a quasi-Poisson error structure for gynes, males, workers and total colony productivity data and GLMs with a logit-link function and a quasibinomial error structure for caste, sex and female-to-male ratios. In all models, *Wolbachia* infection status of the colony was included as a fixed categorical predictor. Models for ratios also included log10-transformed colony total productivity as a continuous predictor. We ran caste and sex ratio models on a data set including all colonies that produced at least one female pupa and on a data set including all colonies that produced at least one sexual pupa, respectively.

Results

Caste ratio

As planned in our bidirectional selection regime, parents in the two replicate high caste ratio selection lines did indeed come from colonies with higher caste ratio values than selected parents in the two replicate low caste ratio lines across generations (Fig. S2 in Appendix S1, one-tailed tests; all $P < 0.05$), confirming that we indeed imposed within-generation artificial selection on caste ratio. However, we observed no between-generation response to selection for caste ratio (Fig. 1a), as offspring colonies in the high and low caste ratio selection lines did not differ across generations (analysis of deviance, overall effect of selection line, $F_{1,337} = 2.68$, $P = 0.10$). There was a small effect of generation ($F_{2,335} = 3.62$, $P = 0.028$) and a strong effect of total colony productivity ($F_{1,334} = 15.52$, $P < 0.001$), confirming that caste ratio is lower (i.e. more workers relative to gynes) in more productive colonies (Schmidt *et al.*, 2011b). All the other terms in the model were nonsignificant (see R scripts in Appendix S3).

Sex ratio

Although we did not observe a significant response to artificial selection for caste ratio, we did observe a

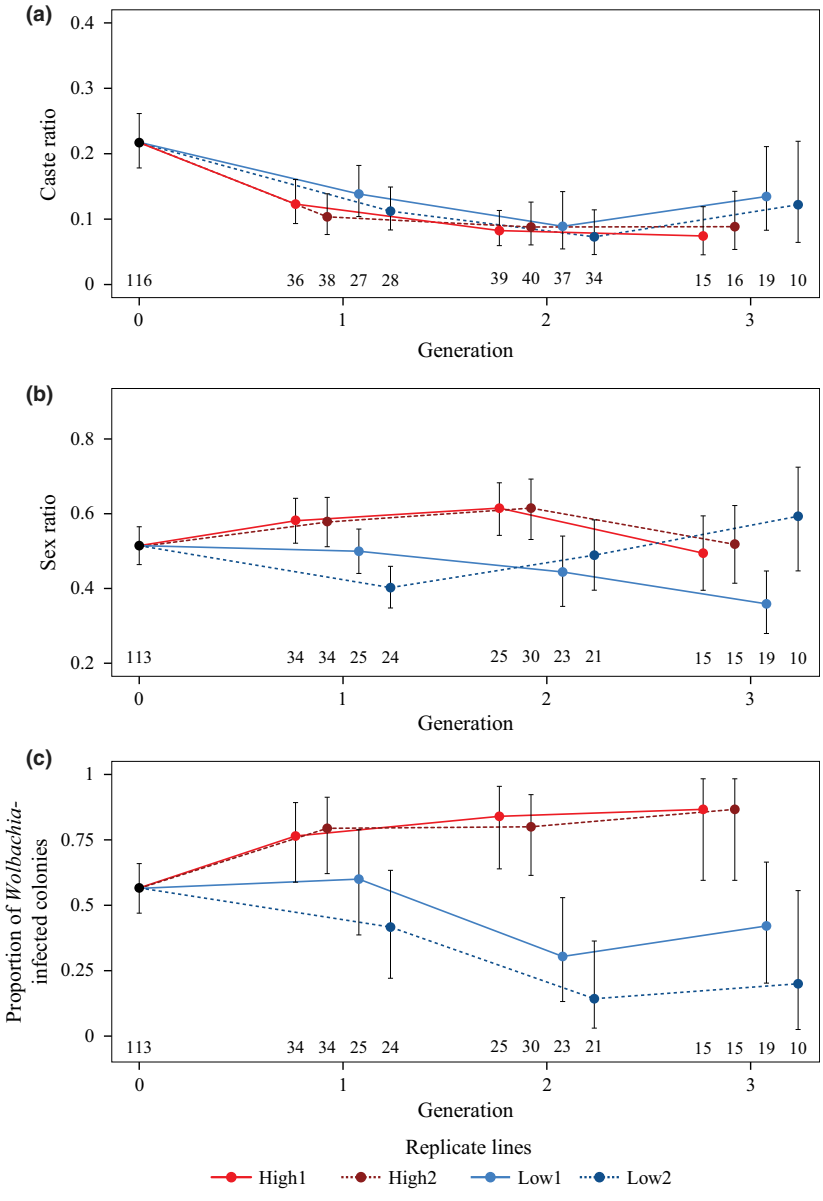

Fig. 1 (a) Caste ratio (defined as gynes/(gynes + workers)) and (b) sex ratio (gynes/(gynes + males)) for replicate high and low selection lines across the three generations of selection (1, 2 and 3) and for the initial breeding population (Generation 0). Dots, colour coded according to replicate line, represent estimates of mean caste ratio (a) or sex ratio (b) based on generalized linear models (GLMs), where total colony productivity was kept at the mean of each replicate line. (c) Proportion of *Wolbachia*-infected colonies for the high and low selection replicate lines across the three generations of selection and for the original breeding population (0). All error bars represent 95% CI. Numbers below each lower bound indicate the sample size of each replicate line.

strong difference between the high and low selection lines for sex ratio across the generations (Fig. 1b; analysis of deviance, overall effect of selection line, $F_{1,273} = 36.85$, $P < 0.001$), with the high selection lines having on average more female-biased sex ratios. There was also a significant interaction between generation and replicate lines nested within selection lines ($F_{4,262} = 3.47$, $P = 0.008$), as the 'Low2' line in the last generation displayed a higher sex ratio value than the other three replicate lines (Fig. 1b). All other terms in the model were nonsignificant (see R scripts in Appendix S3).

Association between *Wolbachia*, selection line and sex ratio

The initial breeding population contained a similar proportion of *Wolbachia*-infected and uninfected colonies (57% infected, Fig. 1c; binomial test: 64 vs. 49, $P = 0.187$). However, the proportion of infected colonies rapidly diverged in the selection lines, with the high and low replicate lines differing for each generation of selection (Fisher's exact tests; Generation 1: $P = 0.01$; Generation 2: $P < 0.001$; Generation 3: $P < 0.001$). Specifically, *Wolbachia* infection became more prevalent in the two high caste ratio lines and less prevalent in the two low caste ratio lines (Fig. 1c).

Consistent with our hypothesis of a female-biasing effect of *Wolbachia* on colony sex ratios, we found that the difference in sex ratio between *Wolbachia*-infected and uninfected colonies in our selection experiment (Fig. 2a; GLM; infected vs. uninfected sex ratio estimates: 0.55 vs. 0.43, $P < 0.001$) was similar both to the difference between colonies in the high and low selection lines after the onset of selection (Fig. 2a; GLM; high vs. low sex ratio estimates: 0.58 vs. 0.45, $P < 0.001$) and to the difference between infected and uninfected colonies in the initial breeding population (Fig. 2b; GLM; infected vs. uninfected sex ratio estimates: 0.57 vs. 0.44, $P < 0.001$), that is before the onset of selection. The sex ratio analysis conducted on each selection line separately further supported our hypothesis, as *Wolbachia*-infected colonies had higher sex ratio values than uninfected colonies, although this difference was only significant for colonies in the high

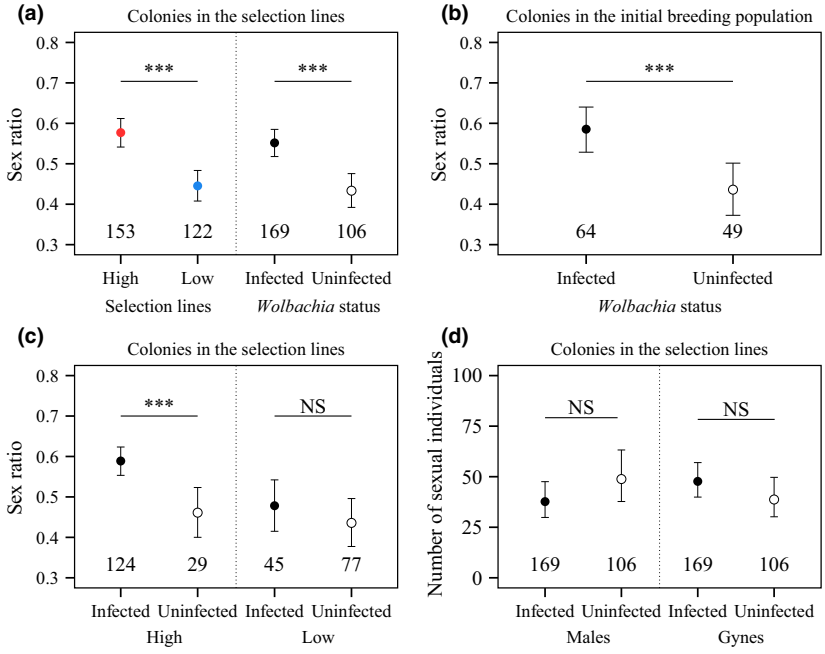

Fig. 2 (a) Sex ratio for colonies after the onset of selection, grouped for selection line (high vs. low; left of dotted line) and for *Wolbachia* infection status (infected vs. uninfected; right of dotted line). (b) Sex ratio for colonies before the onset of selection, grouped for *Wolbachia* infection status. (c) Sex ratio for *Wolbachia*-infected and uninfected colonies after the onset of selection, grouped for selection line (high vs. low). (d) Number of sexuals individuals produced by *Wolbachia*-infected and uninfected colonies after the onset of selection, grouped for sex (males, left of dotted line; gynes, right of dotted line). In (a), dots represent estimates of mean sex ratio based on GLMs where total colony productivity was kept at the overall mean. In (b), dots represent estimates of mean sex ratio based on a GLM where total colony productivity was kept at the mean used in (a). In (c), dots represent estimates of mean sex ratio based on GLMs where total colony productivity was kept at the mean value for each line and infection status. All error bars represent 95% CI. Numbers below each lower bound indicate the sample size (number of colonies) of each group. Significant differences between groups are indicated with asterisks (***<0.001).

selection line (Fig. 2c; infected vs. uninfected sex ratio estimates; high line: 0.59 vs. 0.46, $P < 0.001$; low line: 0.48 vs. 0.44, $P = 0.226$). Altogether, we interpret these results as indicating that the effect of *Wolbachia* on sex ratio was largely independent of the artificial selection regime. Based on fitted values from a GLM, across all colonies in our study (i.e. both before and during selection), infected colonies showed a 0.13 increase in sex ratio compared to noninfected colonies (Fig. 3f; infected vs. uninfected sex ratio estimates: 0.56 vs. 0.43, $P < 0.001$).

The putative female-biasing effect of *Wolbachia* on sex ratio might be the result of an effect on both the number of gynes and the number of males produced. When considering only the colonies in the selection lines, we found that infected and uninfected colonies did not statistically differ for male and gyne production, but infected colonies had a lower mean value for males produced (Fig. 2d; quasi-Poisson GLM, $t_{273} = -0.26$, $P = 0.145$) and a higher mean value for gynes produced (Fig. 2d; quasi-Poisson GLM, $t_{273} = 0.21$, $P = 0.183$) than uninfected colonies. This trend held when we considered all the colonies in our experiment, where we found that infected colonies produced significantly fewer males (Fig. 3b; quasi-Poisson GLM, $t_{454} = -2.17$, $P = 0.030$), and infected colonies tended to produce more gynes, but the difference from uninfected colonies was not significant (Fig. 3c; quasi-Poisson GLM, $t_{454} = 1.49$, $P = 0.136$). Infected and uninfected colonies did not differ for the number of workers produced (Fig. 3d; quasi-Poisson GLM, $t_{454} = -0.2$, $P = 0.845$) and total colony productivity (Fig. 3a; quasi-Poisson GLM, $t_{454} = -0.23$, $P = 0.815$). Infected colonies showed a marginally nonsignificant trend for more gyne-biased caste ratios (Fig. 3e; quasi-Poisson GLM, $t_{452} = 1.96$, $P = 0.051$) and a significantly higher female-to-male ratio (Fig. 3g; quasi-Poisson GLM, $t_{454} = 2.58$, $P = 0.010$).

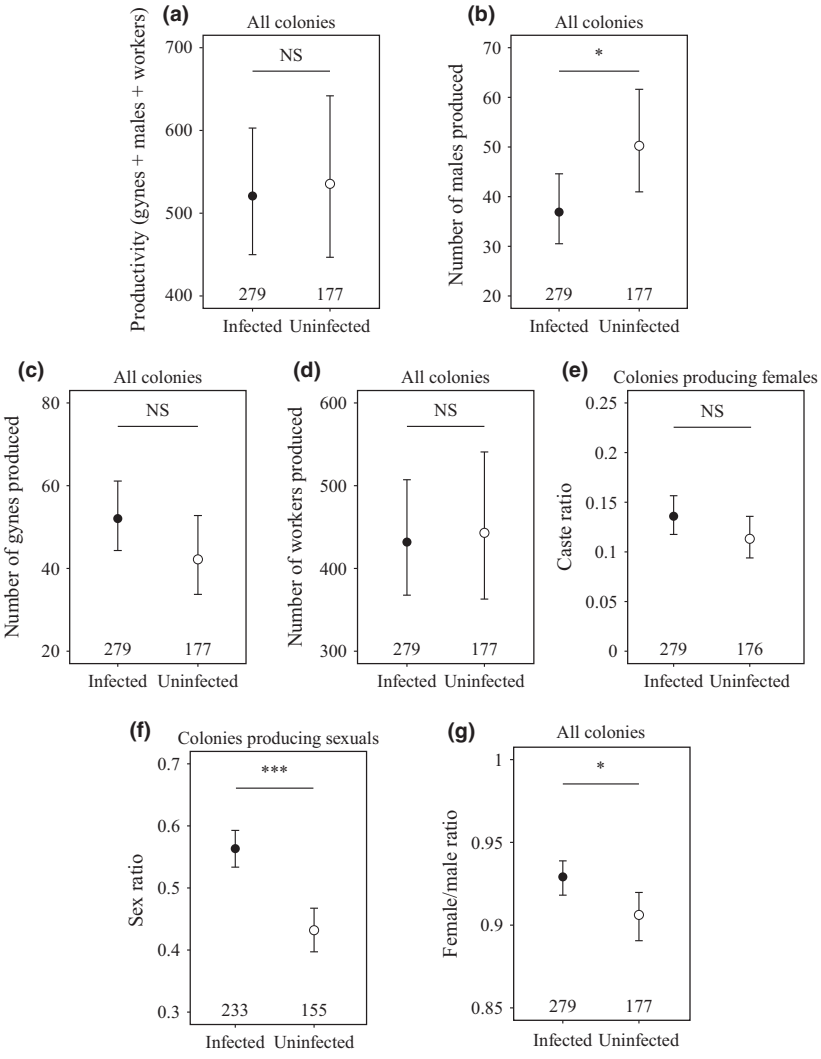

Fig. 3 (a) Total colony productivity, (b) number of males, (c) number of gynes, (d) number of workers, (e) caste ratio, (f) sex ratio and (g) female-to-male ratio for *Wolbachia*-infected (black dot) and uninfected (grey dot) colonies. In (a), (b), (c), (d) and (g), we used all the colonies available in our data set (i.e. all colonies that produced at least one pupa). In (e), we used all the colonies that produced at least one female pupa (i.e. a gyne or a worker). In (f), we used all the colonies that produced at least one sexual pupa (i.e. a gyne or a male). In all graphs, dots represent mean estimates based on GLMs with a quasi-Poisson (a, b, c and d) or a quasibinomial (e, f and g) error structure. In (e) and (f), mean values are those estimated when total colony productivity was kept at its mean value for each group. All error bars represent 95% CI. Numbers below each lower bound indicate the sample size (number of colonies) of each group. Significant differences between groups are indicated with asterisks (*<0.05; ***<0.001).

Discussion

Short-term bidirectional artificial selection on caste ratio in the pharaoh ant *Monomorium pharaonis* did not affect caste ratio, but resulted in rapid divergence for sex ratio and *Wolbachia* infection frequencies between colonies in the high and low selection lines (Fig. 1). We found a similar colony-level association between *Wolbachia* and female-biased sex ratios in our initial breeding population before the start of artificial selection and after the onset of artificial selection (Fig. 2a,b). These results are consistent with the hypothesis that the observed changes in sex ratio across selection lines are primarily driven by a female-biasing effect of *Wolbachia*, rather than by changes in ant genotypic frequencies caused by our artificial selection regime. Our study thus provides the first evidence that *Wolbachia* can manipulate sex allocation in ants, a group of arthropods where the phenotypic effects of *Wolbachia* have previously remained unknown, despite its high prevalence (Andersen *et al.*, 2012; Russell, 2012).

The ability of *Wolbachia* to cause female-biased host sex ratios as a means to favour transmission has previously been reported in several arthropod species (Werren *et al.*, 2008). It has been proposed that similar effects could be present in ants (Chapuisat & Keller, 1999), where *Wolbachia* are widespread, infecting an estimated 30% of all species (Russell, 2012), but the few previous studies that have investigated this hypothesis did not find that *Wolbachia*-infected colonies displayed more female-biased sex ratios (Keller *et al.*, 2001; Wenseleers *et al.*, 2002; Ingram *et al.*, 2012). However, these studies had limited power to detect an effect because either nearly all colonies in the studied populations were infected by a single or multiple strains of *Wolbachia* (Keller *et al.*, 2001; Wenseleers *et al.*, 2002), or few colonies actually produced new gynes and males, precluding a comparison between infected and uninfected colonies (Ingram *et al.*, 2012).

The relatively female-biased sex ratio found in *Wolbachia*-infected colonies in our study resulted primarily from decreased male production, although there was also a trend for infected colonies to have increased gyne production, when compared with uninfected colonies (Fig. 3b,c). *Wolbachia*-infected colonies also showed a trend ($P = 0.051$) to produce more gyne-biased caste ratios (Fig. 3e). Together, the weak gyne bias in caste ratio and the stronger female bias in sex ratio can provide a possible explanation of why infected colonies increased in frequency in the high caste ratio selection lines and decreased in frequency in the low lines across generations.

Further experimental studies, for example using antibiotics to cure colonies infected by *Wolbachia* while controlling for ant genotype, will be necessary to verify the causal role of *Wolbachia* infection and female-biased sex ratios in *M. pharaonis*. The rapid response for sex ratio points to heritable factors of large effect, and the concomitant response in the frequency of *Wolbachia* infection, together with the similar estimated effect in colonies before and during selection (Fig. 2a,b), points to a sex-biasing effect of *Wolbachia*. However, we cannot rule out the possibility that other heritable factors besides *Wolbachia* that are associated with maternal lineage (e.g. ant mitochondrial genes) are at least partly responsible. Moreover, the magnitude of the estimated effect of *Wolbachia* differed between the high and low selection lines (Fig. 2c), suggesting that the effect of *Wolbachia* infection may be conditional on ant genotype or other line-specific environmental factors (i.e. initial queen number, which can have cascading effects on colony productivity, caste ratio and sex ratio, etc.; see Table S3 in Appendix S1 and Schmidt *et al.*, 2011b). Specifically, we note that colonies in one replicate low line ('Low2') in the last generation of selection did not show consistent sex ratio differences compared to the previous generations (Fig. 1b). This result is puzzling, although sample size in this replicate was low (10 colonies) and colonies were also less productive and generally less healthy in this last generation.

Further studies are also required to better elucidate the mechanisms by which *Wolbachia* can influence sex ratio. *Wolbachia* is known to cause at least four distinct classes of reproductive phenotypes in a range of arthropods: (i) feminization, where genetic males develop as females; (ii) parthenogenetic induction, which results in the development of unfertilized eggs into females (thelytokous parthenogenesis); (iii) male killing, where infected males are eliminated to the presumed advantage of the surviving infected female siblings; and (iv) cytoplasmic incompatibility, which prevents infected males from successfully mating with females that lack the same *Wolbachia* type (reviewed in Werren *et al.*, 2008). We can confidently rule out strict cytoplasmic incompatibility because we did not observe an association between parental infection status and total colony productivity (i.e. all combinations of infected and uninfected males and females were fertile). We can also confidently exclude the strict induction of parthenogenesis, because known cases of *Wolbachia*-induced parthenogenesis involve gamete duplication (Wenseleers & Billen, 2000; Rabeling & Kronauer, 2013), and no workers from colonies of the original lineages used to create our study population are completely homozygous (Schmidt *et al.*, 2010). Our results seem to point towards male killing or feminization of genetic males. Both in the selection lines and overall, infected colonies tended to produce fewer males and more gynes than uninfected colonies (Figs 2d and 3b,c).

Besides the four well-studied mechanisms by which *Wolbachia* could manipulate reproductive development, authors have proposed that *Wolbachia* could manipulate queen physiology to affect the likelihood that eggs are fertilized, and thus develop as females (e.g. by

influencing the spermathecal valve that controls access of sperm to eggs; Keller *et al.*, 2001). *Wolbachia* has often been found to localize in the host brain (Dobson *et al.*, 1999; Albertson *et al.*, 2013) and in some insects appears to affect host response to chemical cues (Peng *et al.*, 2008; Furihata *et al.*, 2015). Pharaoh ant workers commonly cannibalize sexual larvae (Edwards, 1991), and *Wolbachia* could influence the behaviour of adult workers, for example by making them more likely to cannibalize male larvae, perhaps in response to larval cues signalling caste or sex. *Wolbachia* might also directly affect the production of these larval cues, which could subsequently influence adult worker provisioning and cannibalization rates.

Heretofore, research on the potential effects of *Wolbachia* on ants, and the underlying mechanisms, has been stymied by the fact that the vast majority of ants cannot be readily bred in the laboratory across generations. Because of the relative ease at which hundreds of *M. pharaonis* colonies can be bred across generations in the laboratory, as well as the broad natural variation in *Wolbachia* infection in *M. pharaonis* (Schmidt *et al.*, 2010), we believe that this ant species represents an exciting model system to further investigate how *Wolbachia* affects ant traits and fitness.

We initially set out to identify heritable variation for caste ratio, with a longer-term goal of identifying causative caste-biasing loci. The observed lack of response to selection for caste ratio indicates that heritability for caste ratio in our study population is undetectable with our study design. These results suggest that levels of segregating additive genetic variation among colonies for caste ratio are low, most consistent with segregating allelic variants being deleterious and maintained at low frequency by mutation–kin selection balance (Van Dyken *et al.*, 2011). Alternatively, most genetic variance for caste may be nonadditive, for example if the caste-biasing effects of alleles depend on genotype at other loci (Schwander & Keller, 2008; Libbrecht *et al.*, 2011). Although our artificial selection study was as large as feasible (consisting of a total of 456 colonies and 158 colonies in the original breeding population), the limited duration (only three generations), and the fact that our design was limited to a single replicate for each colony genotype, may also explain the lack of response to artificial selection for caste ratio. Although we were unable to find evidence for segregating variation in the ant genome for caste ratio, our results do point to a different type of heritable factor with a large effect on sex ratio: the faithfully vertically transmitted endosymbiont *Wolbachia*.

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Appendix S1 Information about the eight original lineages used for the creation of the initial stock of colonies for the selection experiment; crossing scheme and protocol; information about colonies in each replicate line; *Wolbachia* screening protocol.

Appendix S2 Data file (Dryad doi: 10.5061/dryad.f78b1).

Appendix S3 R scripts used for statistical analyses and figures (Dryad doi: 10.5061/dryad.f78b1).

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