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Bacterial Endosymbionts Are Common Among, but not Necessarily Within, Insect Species

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Abstract

Bacterial endosymbionts, particularly *Wolbachia* (Rickettsiales: Rickettsiaceae), *Rickettsia* (Rickettsiales: Rickettsiaceae), and *Cardinium* (Bacteroidales: Bacteroidaceae), are commonly found in several arthropod groups, including insects. Most estimates of the global infection rate of *Wolbachia* (52% [95% credible intervals: 44–60]) show that these bacteria infect more than half of all insect species. Other endosymbionts, such as *Rickettsia* (24% [confidence intervals [CIs] 20–42]) and *Cardinium* (13% [CIs 13–55]), infect a smaller but still substantial proportion of insect species. In spite of these observations, it is unclear what proportion of individuals *within* those species are infected. Here, we used published databases to estimate the proportion of individuals that are infected with either *Wolbachia*, *Rickettsia*, or *Cardinium*. We found that the majority (69%) of *Wolbachia*-infected species have less than half of their individuals infected with *Wolbachia*, indicating that although the bacterium may be common among species, it is not common within species. The same was true for *Rickettsia* (81%) and *Cardinium* (87%). This discrepancy was consistent across orders, in which less than 10% of individuals were typically infected, even though more than 50% of species within orders were infected. For example, according to our model, nearly 50% of beetle (Coleoptera) species are infected with *Wolbachia* (i.e., contain at least one individual that has tested positive for *Wolbachia*), but less than 5% of all individuals are infected. These results add to the growing knowledge base about endosymbionts in insects and should guide future sampling efforts and investigations on the role that these bacteria play in populations.

Key words: *Wolbachia*, population, Bayesian, endosymbiont, insect

Endosymbionts, such as *Wolbachia*, *Rickettsia*, and *Cardinium*, are a type of bacteria commonly found in arthropod species including insects, spiders, crustaceans, and mites, as well as in other invertebrates such as filarial nematodes (Stouthamer et al. 1999, Moran 2006, Werren et al. 2008, Engelstädter and Hurst 2009). Although these bacteria are not the only reproductive manipulators (Engelstädter and Hurst 2009), they are currently the most well studied. They are commonly referred to as reproductive manipulators due to the phenotypes they exhibit on their hosts. *Wolbachia* is the most prominent member of the reproductive manipulators due to its wide range of phenotypes and the large number of species it infects. It has been estimated that *Wolbachia* infects 52% (95% CI 44–60%) of all insect species (Sazama et al. 2017), whereas *Rickettsia* and *Cardinium* infect 24% (20–42%) and 13% (13–55%) of all arthropod species, respectively (Weinert et al. 2015).

Bacterial endosymbionts can infect a high proportion of individuals within some species (Jiggins et al. 2001), but it is unclear whether there is systematic variation in the proportion of infected individuals across taxa. Most analyses of *Wolbachia* infection and other reproductive manipulators have focused on the proportion of species infected with these symbionts, rather than the proportion of individuals within a species (Hilgenboecker et al. 2008, Zug and Hammerstein 2012, Weinert et al. 2015, Sazama et al. 2017). Studies on the proportion of infected individuals are typically restricted to simply sampling and reporting the presence of *Wolbachia* in randomly sampled insects with hardly any statistical analysis of the data (Werren et al. 1995, Werren and Windsor 2000, de Albuquerque et al. 2011, Wiwatanaratanabutr and Zhang 2016). For example, Wiwatanaratanabutr and Zhang (2016) found that 100% (508/508) of *Aedes albopictus* (Skuse) (Diptera: Culicidae) were infected with *Wolbachia*. According to a study by de Albuquerque et al (2011), 91.61% (131/143) of *Ae. albopictus* were infected with *Wolbachia*. The differences in these two samples are not large, but they suggest that there is some degree of variability with regard to *Wolbachia* infection that may depend on where and when samples were collected. Therefore, a substantial analysis is needed to estimate these proportions and analyze the amount of uncertainty associated with the estimates. Doing so will guide future sampling efforts and

improve estimates on the overall abundance of endosymbionts in insects. The focus of this work is to understand the infection frequencies within species for endosymbionts of insects (*Wolbachia,* *Rickettsia,* and *Cardinium*).

Endosymbionts affect their hosts in a variety of ways. For example, *Wolbachia* is often characterized as a reproductive manipulator because of the effects it has on insects (Werren 1997, Werren et al. 2008). These effects include parthenogenesis, cytoplasmic incompatibility, feminization, and male-killing. In parthenogenesis, *Wolbachia* induces all-female lines of the host (Stevens et al. 2001, Werren et al. 2008). Feminization and male-killing create selection pressures to reallocate resources from males to females (Stevens et al. 2001, Kageyama et al. 2002, Negri et al. 2010). Cytoplasmic incompatibility involves a manipulation of the sperm and egg by *Wolbachia* to induce an incompatibility between an infected male and uninfected female (Tram and Sullivan 2002, Rasgon and Scott 2003, LePage et al. 2014). *Cardinium* is also known to exhibit cytoplasmic incompatibility, feminization, and parthenogenesis (Bourtzis and Miller 2006, Engelstädter and Hurst 2009), whereas *Rickettsia* can show male-killing and parthenogenesis (Perlman et al. 2006). The previously mentioned reproductive effects either improve host fitness or cause harmful reproductive manipulations (Zug and Hammerstein 2017).

Reproductive manipulations can affect the biology of the host and can potentially affect the population size of the host. For example, Mains et al. (2013) found that *Wolbachia*-induced mortality caused a reduction in the population size of infected mosquitos (69.1 vs 60.8% survival in females and 47.6 vs. 37.4% survival in males for uninfected and infected populations, respectively). Moreover, *Wolbachia* reduced oviposition in infected mosquitos by at least 30% (Mains et al. 2013). Similar reductions in fecundity and fitness also occur with *Cardinium* (Perlman et al. 2008) and *Rickettsia* (Sakurai et al. 2005). In natural populations, the importance of *Wolbachia*-induced mortality (or reduced reproductive output) in regulating population sizes would depend on the proportion of infected individuals *within* species. For example, a 50% mortality rate would potentially devastate a population in which nearly all individuals are infected (as seen in Rasgon and Scott 2003) but have very little effect on a population with a proportion of infected individuals <1% (as seen in Tagami and Miura 2004). Due to the concerns in the use of *Wolbachia* as a biocontrol (Loreto and Wallau 2016, but see Dobson et al. 2016, O'Neill 2016), assessments of the infection frequencies among and within natural populations of species are critical for risk assessment.

Here, we estimate the proportion of endosymbiont-infected individuals within 88,153 tested individuals across 3,370 species. We tested the hypothesis that endosymbionts are common *among* species, but not necessarily *within* species, and that this disparity is consistent across insect orders.

Materials and Methods

Database

Previously published databases were used to estimate the proportion of infected individuals within infected species (Zchori-Fein and Perlman 2004, Duron et al. 2008, Weinert et al. 2015, Sazama et al. 2017). These databases were chosen to provide direct comparisons between the infection frequencies found *among* species in those papers and the infection frequencies *within* species found in this analysis. First, noninsect species were removed (1,829). We then removed samples that did not identify beyond the order level (224 rows of data). The resulting database, after filtering, includes 88,153 tested

individuals representing 3,370 species, 319 families, and 24 orders. The number of species tested for *Wolbachia* was 2,766 (62,641 individuals). The number of species with only one individual tested was 1,526. The number of species tested for *Rickettsia* was 747 (19,686 individuals). The number of species tested for *Cardinium* was 1,015 (5,826 individuals). For *Rickettsia,* the number of species with only one individual tested was 288 species. For *Cardinium,* the number of species with only one individual tested was 690 species. Some of the species were tested for all three bacteria, some with a mixture of two of the bacteria, but the majority tested with only one bacterium. The full Sazama database is available online at doi:10.5063/F1, and the full Weinert database is available online at doi:10.1098/rspb.2015.0249.

Model

To determine the proportion of infected individuals within species, we used an intercept-only generalized linear mixed model with a binomial likelihood. The response variable was the number of individuals infected per sample (out of the total tested). Species was included as a random effect, producing an estimate of the proportion of infected individuals within each species in the database. We ran separate models for *Wolbachia* (2,766 species), *Rickettsia* (747 species), and *Cardinium* (1,015 species). In addition to the species-level analysis, we also modeled the proportion of individuals infected within orders. To do this, we again used a generalized linear mixed model with a binomial likelihood and the same response variable. We included order as a fixed predictor variable and species as a random effect. We ran both models on the full data set described earlier. However, this data set contained a large number of samples with only a single individual. To determine whether results were affected by small samples, we reran the second model (i.e., the order model) with a data set that only contained samples in which more than five individuals were tested. The criteria for only including species which have more than five individuals tested lies in a drop in the amount of uncertainty associated with a larger sample size (Supp Figs. 1–5 [online only]). In addition to these models, we ran a similar model to that used in Sazama et al (2017), which was a generalized linear model with a beta-binomial likelihood that estimated the proportion of species infected with an endosymbiont. In addition, we analyzed the potential coinfection of endosymbionts in the tested taxa (Supp Fig. 6 [online only]). We analyzed coinfection between all three groups of bacteria and between each group separately. There were 113 species for which all three endosymbionts were tested. There were 121 species for which *Wolbachia* and *Cardinium* were both tested. There were 164 species for which both *Wolbachia* and *Rickettsia* were tested. There were 113 species for which both *Cardinium* and *Rickettsia* were tested.

The models above produced a value between 0 and 1 for the proportion of infected individuals of each insect species or order. However, the values only asymptotically approach 0 and 1. For this reason, we used a cutoff of 0.001 to categorize a species as infected (>0.001) or uninfected (<0.001), following precedent from other analyses of bacterial endosymbiont infections (Hilgenboecker et al. 2008, Zug and Hammerstein 2012).

All models were specified in R (Version 3.4.1) using *rethinking* (McElreath 2016) or *brms* (Burkner 2017) packages. The posterior distribution was generated using Hamiltonian Monte Carlo via *rstan* (Stan Development Team 2018). The validity of all models was assessed visually by examining the convergence of trace plots and by noting that r-hat was less than 1.1. All models met these criteria and were considered valid. All code for the model and figures is posted to github at https://github.com/ericjsazama/withinspecies.

Results

For *Wolbachia*, 488/622 species in which more than five individuals were tested were considered infected. Within the infected species, the majority (69%) were infected below a proportion of 0.5. Of the 54/287 species in which more than five individuals were tested and considered infected for *Rickettsia*, we found that the vast majority (81%) were infected below a proportion of 0.5. In addition, 31/105 species with more than five individuals tested for *Cardinium* were considered infected, and, as with *Rickettsia*, the vast majority (87%) were found to be infected below a proportion of 0.5 (Fig. 1). This is reflected in the S-shaped distribution when the proportion of individuals infected is plotted in order of lowest to most highly infected species (Fig. 1). This pattern was consistent across the different bacteria (Fig. 2) and different orders (Supp Figs. 1–3 [online only]). There was no discernible trend for coinfection with multiple bacteria for each species, though different detection methods and a lack of consistent sampling and testing for all bacteria make it difficult to see any trends.

Among orders infected with *Wolbachia*, the proportion of individuals infected varied from 0.2 (order Ephemeroptera) to 76% (Siphonaptera). It was below 5% in 19/24 orders (Fig. 3). When the analysis was restricted to only samples with more than five individuals, the mean proportion of infected individuals varied between 0.5 and 28% (Fig. 3). The proportion of *species* infected within orders (14 and 99%) was far greater than the proportion of *individuals* infected for all bacteria and orders (Fig. 3).

Among orders infected with *Rickettsia*, the proportion of individuals infected varied from 0.01 (Hymenoptera) to 7.8% (Diptera). It was below 5% in 13/14 orders (Fig. 3). When the analysis was restricted to only samples with more than five individuals, the mean proportion of infected individuals varied between 0.02 and 4% (Fig. 3). The proportion of *species* infected within orders (10 and 96%) was far greater than the proportion of *individuals* infected for all bacteria and orders (Fig. 3).

Among orders infected with *Cardinium*, the proportion of individuals infected varied from 0.004 (Lepidoptera) to 0.8% (Hemiptera). It was below 5% in all 17 orders (Fig. 3). When the analysis was restricted to only samples with more than five individuals, the mean proportion of infected individuals varied between 0.5 and 25% (Fig. 3). The proportion of *species* infected within orders (2 and 66%) was far greater than the proportion of *individuals* infected for all bacteria and orders (Fig. 3).

Coinfection among groups did occur (Supp Fig. 6 [online only]). However, this event appears to be rare because the majority of species in the figure are considered uninfected or at a low infection rate. There are a few instances in which coinfection with one group appears to lower the presence of the bacteria in another group (e.g., *Cardinium* lowers the infection with *Wolbachia* in a few species). *Wolbachia* is still the most prevalent bacteria, but this figure suggests that *Wolbachia* may have to compete with the other bacteria.

Discussion

Endosymbiont infection of insect species has been well documented (Hilgenboecker et al. 2008, Zug and Hammerstein 2012, Weinert et al. 2015, Sazama et al. 2017). However, there is a relative lack of knowledge of infection frequencies within species. Here, we sought to determine such frequencies to better understand the distribution of endosymbionts within species. The results demonstrate that although 52% of insect species are infected with *Wolbachia*, most species have infection frequencies well below 50% (Fig. 1), meaning that *Wolbachia* is widespread among taxa, but not necessarily widespread among individuals within taxa. The same is also true of *Rickettsia* and *Cardinium*.

Several mechanisms could explain the maintenance of low proportions of infected individuals across species. For example, it is possible that bacterial endosymbionts have low infection frequency until there is a positive selective pressure. A positive selective pressure would then push the prevalence of the endosymbiont in that species to 100%. An example of such a pressure is mutual symbiosis, in which the reproductive manipulator evolves traits to increase the hosts' fitness (Brucker and Bordenstein 2012, Zug and Hammerstein 2015). An alternative explanation for low infection frequencies is that endosymbiont infection is transient, meaning that the bacteria move in and out of populations readily (Siozios et al. 2018, Turelli et al. 2018). Recent estimates of gain and loss of *Wolbachia* over time indicate that *Wolbachia* incidence has reached an epidemiological
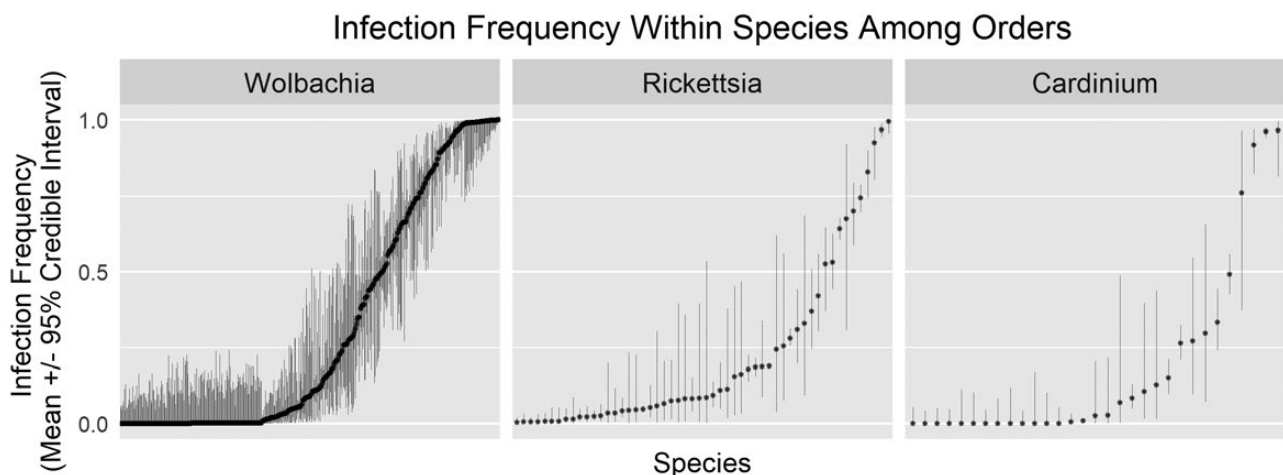
Fig. 1. Endosymbiont infection frequencies in insect species where greater than five individuals were tested. These values also consist of only infected species (proportion of infected > 0.001). Each dot represents a specific species that has been tested within the literature for these bacteria. There are 488, 54, and 31 species that meet the criteria in this graph for *Wolbachia*, *Rickettsia*, and *Cardinium*, respectively. The y-axis and each corresponding value represent the mean of the posterior distribution plus/minus the 95% credible interval. This model estimates the infection frequency within species based on the existing data. The proportion of individuals below the infection frequency of 0.5 is 0.69 for *Wolbachia*, suggesting that the majority of insects are infected at a low frequency. The same observation is true for *Rickettsia* (0.81) and *Cardinium* (0.87).

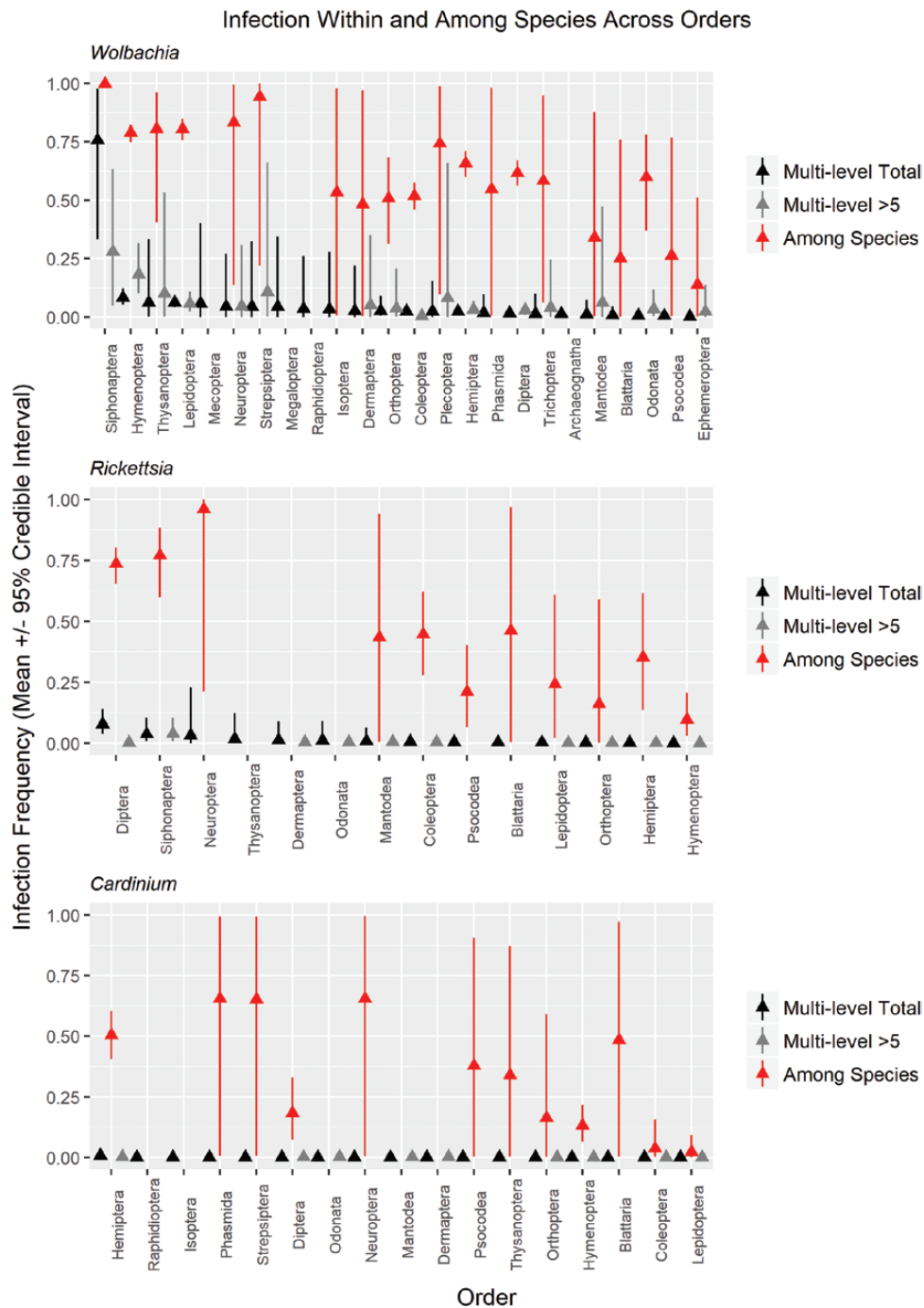## Infection Frequency Within Species of Diptera Among Bacteria
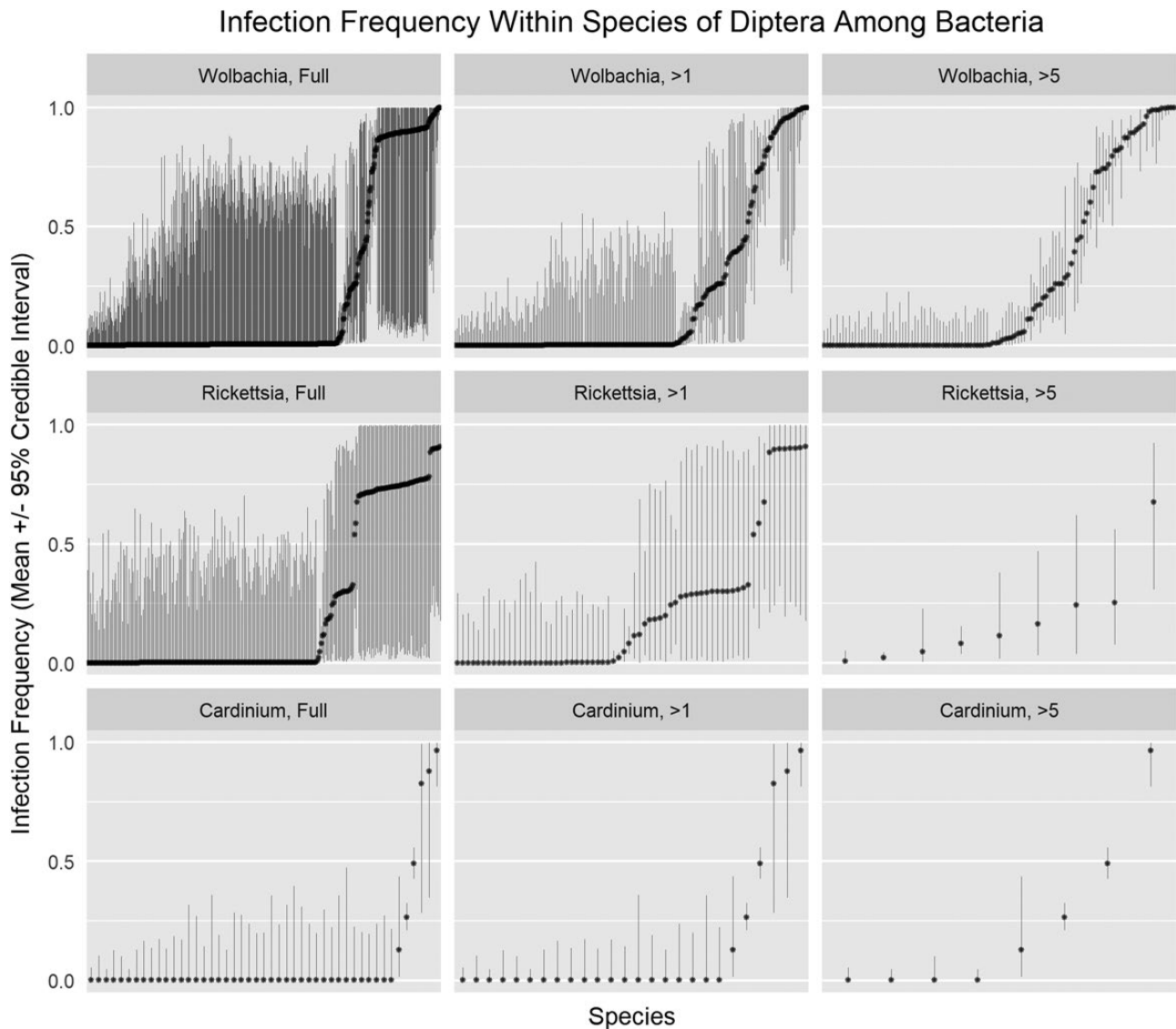
Fig. 2. Infection frequencies of species based on the mean of the posterior distribution plus/minus the 95% credible interval for each of the endosymbionts. Diptera were chosen to represent these data because the presence of all three bacterial genera was tested within several species. The results were then further divided into relative sampling efforts, as noted by the respective labels. For instance, '*Wolbachia*, Full' contains the entire database of infected species (infection frequency > 0.001), whereas '*Wolbachia*, >5' only contains infected species for which more than five individuals were sampled. This graph serves as a visual indicator for how the uncertainty varies between sampling efforts.

equilibrium (Bailly-Bechet et al. 2017). An equilibrium state would involve a constant state of gain and loss of *Wolbachia* among species, which would also explain the low abundance within infected species seen here. Potentially, competition with other bacteria may interfere with the presence of an endosymbiont just as *Wolbachia* can compete with other bacteria for resources within the host (Ye et al. 2013). In addition, *Wolbachia* can live outside of the germline, potentially allowing the bacteria to persist without disrupting the reproductive biology of their host (Pietri et al. 2016). In this study, it is unclear whether the high number of species with low to intermediate proportions of infected individuals represents transient endosymbiont infections or a lack of established infection within these species.

The overall pattern of a skewed S-shaped distribution of the proportion of infected individuals was consistent across orders (Supp Figs. 1–3 [online only]), suggesting a common pattern in which most species have low infection frequencies, regardless of order. The only minor exception to the pattern of infection is Hymenoptera infected with *Wolbachia*, in which there appear to be more species in the intermediate and higher ranges of infection frequencies. This difference could be due to their parthenogenic and other interesting reproductive methods further enhanced by their mechanisms for sex determination (Cook and Crozier 1995, Ahmed et al. 2013). There are also several infected groups within the Formicidae, with over 3,500 individuals tested for one species (*Solenopsis invicta*) of this group. The Formicidae are eusocial insects and present a unique route for endosymbiotic bacteria (Russell 2011). This order also has a large number of parasitoid and parasitic species that could be infected more so than other species (Huigens et al. 2004, Ahmed et al. 2015, Pietri et al. 2016). Of the 39 hymenopteran families tested for *Wolbachia*, 21 are either fully parasitic/parasitoid or at least have several members that are parasitic/parasitoid on other insects. The sex determination for many Hymenopteran species also leaves them vulnerable to parthenogenesis with *Wolbachia* and other endosymbionts (Cook and Crozier 1995, Russell 2011).

Fig. 3. Comparison of the infection frequencies among species and among individuals across the different orders and different bacteria represented in this study. The leftmost triangles in an order represent the proportion of infected individuals within orders as obtained through a multilevel model, the middle triangles for an order represent the frequency among species obtained by using similar models from Sazama et al. (2017), and the rightmost triangles for an order represent the proportion of infected individuals within species where more than five individuals were tested. Each object represents the mean of the posterior distribution plus/minus the 95% credible interval. For orders where there was only one species tested or if there were too few individuals tested, the representative symbol is missing as there were no results for that particular order.

In addition, the number of low infection frequencies decreases as more insects are sampled (moving from left to right in Fig. 2), possibly pointing toward a bias in large sampling efforts toward taxa known to be previously infected (Zug and Hammerstein 2012). This could potentially elevate the estimates of infection because the species that are tested the most will influence the model and lead to higher estimates of infection. Typically, the estimates of overall

infection incidence are considered to be underestimated, as mentioned previously due to low sample sizes (Weinert et al. 2007, 2015). Therefore, a small sample size will underestimate endosymbiont infection, whereas a concentrated sampling of infected species will overestimate endosymbiont infection. In a similar way, if sample sizes are small, but all the samples test positive, then endosymbiont infection will be overestimated. There is evidence for both biases,

and together they reinforce the need for more sampling and testing for the presence of endosymbionts within insect species. However, the estimates in this analysis are based on the current data and represent the infection frequencies using confidence intervals that partially reflect sampling biases in the uncertainty intervals.

At the order level, the proportion of infected individuals (Fig. 3 black triangles) was uniformly low across all bacteria, with the exception of Siphonaptera infected with *Wolbachia* (~75% of individuals infected). However, this high value is partially inflated because many of the infected samples tested within this order had only one individual. To test whether the larger proportion of infected individuals was due to a sampling size problem, we ran the same model but excluded samples where five or less individuals were tested. The result lowered the proportion of infected individuals (~25%) within Siphonaptera (Fig. 3, gray triangles), the most infected order. We suspect that this group of insects may be particularly susceptible to *Wolbachia* and other symbiote infection due to their mechanism of feeding. For example, Siphonaptera are liquid-feeding ectoparasites, and liquid-feeders from other orders can obtain *Wolbachia* directly from their diet (Sintupachee et al. 2006, Li et al. 2017). The previous examples were plant-mediated but open the possibility for a similar mechanism in blood-feeders.

Our results also highlight the differences between measuring *Wolbachia* infection across species versus among individuals. For instance, the Strepsipterans have a very low proportion of infected individuals within species but have a very high proportion of species infected. This effect remains even when the same model is limited to sample sizes greater than five individuals. In this case, we would probably not see a large influence on the population of Strepsipterans by *Wolbachia* because they are not infecting a large proportion of individuals within the order, even though most of the species are considered infected. The same pattern of infection can be seen throughout many of the major insect orders. For instance, *Wolbachia* infects approximately 50% of species in Coleoptera, yet infects less than 10% of individuals within the order. This pattern is consistent among Hemiptera, Diptera, and several other orders (Fig. 3). The estimates of *Wolbachia* incidence among species may differ from other estimates of the incidence of *Wolbachia* among species. For instance, Kajtoch and Kotásková (2018) found that 38% of Coleoptera species have *Wolbachia*. The main reason for these differences in estimates is probably due to a difference in the method of estimating incidence. In any case, *Wolbachia* is highly prevalent among species of Coleoptera, yet not nearly as prevalent among individuals within Coleoptera. This further accentuates the point of this work that *Wolbachia*, and potentially other endosymbionts, are common among species, but not necessarily within species of insects.

The relatively low proportion of individuals that are infected within most species suggests that bacterial endosymbionts, though widespread taxonomically, are likely to have minimal effects on population abundances of most species. As a result, even if wild insect populations were infected with a strain of endosymbiont that altered survival rates (Mains et al. 2013), two conditions would probably need to be met for the endosymbiont to cause effects at the community or ecosystem level: 1) the insect taxon would need to be abundant and 2) the proportion of infected individuals in the population would need to be high. Otherwise, any effects of endosymbionts on ecological processes would be swamped by more common drivers of ecological function, such as nutrient limitation, predation, or disturbance.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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