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INVERTEBRATE MICROBIOLOGY

Development of Common Leaf-Footed Bug Pests Depends on the Presence and Identity of Their Environmentally Acquired Symbionts

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ABSTRACT Many beneficial symbioses between bacteria and their terrestrial arthropod hosts are vertically transmitted from mother to offspring, ensuring that the progeny acquire necessary partners. Unusually, in several families of coreoid and lygaeoid bugs (Hemiptera), nymphs must instead ingest the beneficial symbiont, *Burkholderia* (*sensu lato*), from the environment early in development. We studied the effects of *Burkholderia* on development of two species of leaf-footed bug (Coreidae) in the genus *Leptoglossus*, *Leptoglossus zonatus* and *Leptoglossus phyllopus*. We found no evidence for vertical transmission of the symbiont but found stark differences in performance between symbiotic and aposymbiotic individuals. Symbiotic nymphs grew more rapidly, were approximately four times more likely to survive to adulthood than aposymbiotic bugs, and were two times larger. These findings suggest that *Burkholderia* is an obligate symbiont for the *Leptoglossus* species. We also tested for variation in fitness effects conferred by four symbiont isolates representing different species within the *Burkholderia* insect-associated stinkbug beneficial and environmental (SBE) clade. While three isolates conferred similar benefits to hosts, nymphs associated with the fourth isolate grew more slowly and weighed significantly less as adults. The effects of the four isolates were similar for both *Leptoglossus* species. This work indicates that both *Burkholderia* acquisition and isolate identity play critical roles in the growth and development of *Leptoglossus*.

IMPORTANCE *Leptoglossus zonatus* and *L. phyllopus* are important polyphagous pests, and both species have been well-studied but generally without regard to their dependance on a bacterial symbiont. Our results indicate that the central role of *Burkholderia* in the biology of these insects, as well as in other leaf-footed bugs, should be considered in future studies of coreid life history, ecology, and pest management. Our work suggests that acquisition of *Burkholderia* is critical for the growth and development of *Leptoglossus* species. Further, we found that there was variation in performance outcomes according to symbiont identity, even among members of the stinkbug beneficial and environmental clade. This suggests that although environmental acquisition of a symbiont can provide extraordinary flexibility in partner associations, it also carries a risk if the partner is suboptimal.

KEYWORDS horizontal transmission, *Burkholderia*, gut symbionts, host-symbiont interactions, symbiosis

Animals frequently rely on microbial partners to provide nutrients that are limiting in their diets (1, 2), for defense (3, 4), or for detoxification of environmental or diet-associated toxins (5, 6). In terrestrial animals, these beneficial microbial associates are often transmitted vertically from mother to offspring, either within the egg cytoplasm,

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through the birth process, on the egg surface, or via special maternal secretions (7–10). Other beneficial symbionts may be transmitted vertically or horizontally via proctodeal feeding or social interactions within and between generations (11–13).

In terrestrial arthropods, vertically transmitted symbionts often take up residence within cells in specialized host organs. These associations are evolutionarily durable, some of them hundreds of millions of years old (14, 15). Over time, however, the genomes of strictly vertically transmitted symbionts become degraded and reduced, due to both population bottlenecks associated with transmission and relaxed selection on biosynthesis of redundant products made by both host and symbiont (15). Therefore, many nutritional symbionts' genomes contain only a minimal set of genes transcribing the products that either symbiont or host requires (15). These intracellular microbes are reliably transmitted from parent to offspring and benefit the insect in defined ways but may have limited capacity to respond to new environmental challenges.

In contrast to these long-lasting associations, a recently discovered association between free-living bacteria in the genus *Burkholderia* (*sensu lato*) and several families of coreoid and lygaeoid bugs in the Hemiptera (16, 17) provides a unique alternative model for beneficial symbiosis in an insect host. Bug-*Burkholderia* associations appear to persist predominantly or entirely via environmental transmission, similar to many marine animal symbioses (18–20). Bug females do not transmit *Burkholderia* to their offspring. Instead, young nymphs must find and ingest the bacterium, after which the symbiont colonizes and occupies a specific midgut region, the fourth midgut section or M4 (17). While the nutritional benefits provided by *Burkholderia* are not easily discerned from the large (~6.5 Mbp) genomes of these free-living organisms, a comparative transcriptome analysis of *Burkholderia* in the insect versus in culture suggested that symbiont services include nitrogen recycling and amino acid and B vitamin biosynthesis (21). In each host species studied, dozens to hundreds of strains of *Burkholderia* have been found, with most belonging to a single group, the stinkbug beneficial and environmental (SBE) clade (17, 22–24). Recently this major *Burkholderia* clade has been split off and placed in *Caballeronia* (25) with other clades also receiving new genus level classifications (26). Given that coreoid and lygaeoid Hemiptera associate with strains across the genus *Burkholderia sensu lato*, in this study we will refer to all hemipteran symbionts as *Burkholderia*, and the primarily Hemiptera-associated clade examined here as the SBE clade.

In the model system for the exploration of hemipteran-*Burkholderia* associations, the soybean-feeding *Riptortus pedestris* (Alydidae) bugs acquire the symbiont from the soil, primarily as second instar nymphs (27). Ingested *Burkholderia* colonizes crypts in the M4 gut region. Following colonization, a constricted region between the M3 midgut section and the anterior M4B region seals, cutting off access to the symbiotic region (28). *Burkholderia* grows to high density in the M4 and cells flow backward into the anterior M4B region, where the bacteria are digested and their products absorbed (29). *Riptortus pedestris* nymphs that acquire the symbiont are larger as adults, develop more quickly, and lay more eggs, indicating the benefit of the symbiont to the bugs (16, 30, 31). Investigations of other host species with *Burkholderia* symbionts suggest that the general features of the *R. pedestris-Burkholderia* symbiosis are shared broadly across host taxa (17, 22–24, 32–35), but few other systems have been investigated in detail.

The leaf-footed bug genus *Leptoglossus* (Coreidae) is common in North and South America. *Leptoglossus zonatus* is a pest of fruits and developing seeds with a geographic range that includes the Western and Southeastern United States, Central America, and Brazil (36–38). In recent years, the range of this insect has expanded (39). In California, it is an important pest of pomegranates and an occasional pest of almonds and pistachios as well as in home gardens (40). This species is also an increasing problem on tomato and citrus in the Southeast (41, 42). In the Eastern United States, *Leptoglossus phyllopus* fills a similar ecological role to that of *L. zonatus*. This species is also widely polyphagous,

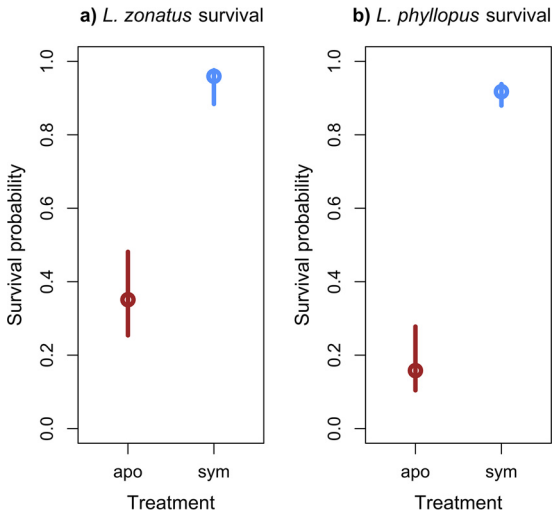

FIG 1 Survivorship of second instar *Leptoglossus* nymphs to adulthood in aposymbiotic ("apo") versus symbiotic ("sym") treatments. Plotted are model probabilities that a second instar *L. zonatus* or *L. phyllopus* nymph survived to adulthood based on symbiont colonization status. Estimates are based on one mixed logistic regression model for each insect species, controlling for cage as a random effect in both cases. Circles show estimated group means; error bars show estimated standard errors (the standard errors are asymmetric because the model was on a logit scale, but the *y* axis is not).

feeding on fruits, including peaches, and vegetable and field crops, such as cotton, cowpeas, sorghum, faba bean, and tomatoes (36, 43–45).

Despite their status as relatively common and economically important pests, the fact that *Leptoglossus* species are likely to have beneficial *Burkholderia* symbionts that may influence their fitness has been overlooked. In this paper, we address the following questions. Is *Burkholderia* important to the performance of these two widespread *Leptoglossus* species? In these coreid pests, is environmental transmission of *Burkholderia* required, or does vertical transmission play a role in symbiont acquisition? The latter might be expected for insects whose delicate, flightless nymphs often live in trees, far above the soil from which the symbiont can be reliably acquired. Lastly, do different *Burkholderia* SBE clade isolates have similar effects on bug performance? Flexibility in partner association is a unique aspect of environmental acquisition of symbionts that should have important implications for symbiont function and host fitness if symbiont strains are differentially beneficial and/or locally adapted to environmental conditions (46).

RESULTS

Role of symbiont infection in bug development and performance. We asked whether the developmental success of aposymbiotic *Leptoglossus* bugs differed from those fed *Burkholderia* in the second instar. The differences between treatments were dramatic. Most bugs reared without access to *Burkholderia* died before adulthood (*L. zonatus*, 62%; *L. phyllopus*, 84%) while only 6% and 8% of the symbiotic bugs did, respectively. After accounting for replicate cage effects (in which nymphs were reared in groups), *L. zonatus* nymphs infected with *Burkholderia* were 3.8 times more likely to survive to adulthood than aposymbiotic nymphs (Fig. 1) ($df = 1$, $\chi^2 = 14.1$, $P = 0.0002$). Similarly, *L. phyllopus* nymphs were 4.1 times more likely to survive to adulthood (Fig. 1) ($df = 1$, $\chi^2 = 23.7$, $P < 0.0001$).

The aposymbiotic bugs that did survive took much more time to reach adulthood than the symbiotic bugs ($df = 1$, $\chi^2 = 27.3$, $P < 0.0001$) (Fig. 2). After accounting for the random effect of cage, aposymbiotic *L. zonatus* bugs took 12 days longer (~50% longer) than symbiotic bugs to reach adulthood. Aposymbiotic *L. phyllopus* bugs were even slower, taking 25 days longer (~90% longer) than symbiotic individuals. Within

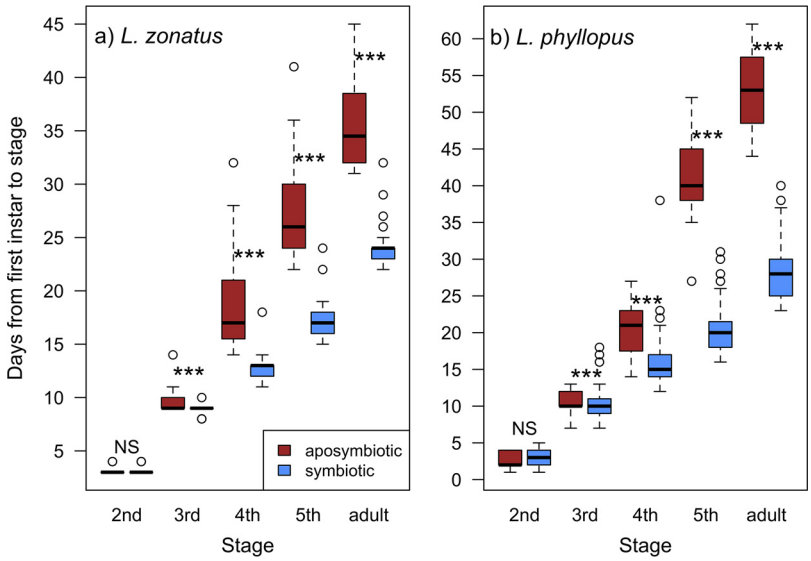

FIG 2 Development time of *Leptoglossus* in aposymbiotic versus symbiotic treatments (from beginning of first instar to stage). Boxplots show the raw data from all insects, including those that died as nymphs. At each stage after the second instar, aposymbiotic bugs (red bars) of both species took significantly longer to develop than symbiotic bugs (blue bars). ***, $P < 0.001$.

each sex (females are naturally heavier than males), aposymbiotic insects that reached adulthood had fresh weights of approximately half that of their symbiotic counterparts at eclosion (Fig. 3).

The relatively few aposymbiotic *L. zonatus* individuals that reached adulthood were, moreover, more likely to have cuticular deformities (Fig. 4). Ten of the 20 total adults that eclosed in the aposymbiotic treatment either had trouble molting or had noticeable deformities, including wing deformities, attached fifth instar cuticle, and sex ambiguity. Only three aposymbiotic *L. phyllopus* survived to adulthood. None of these were deformed, but the sample size is small.

Vertical transmission of *Burkholderia* in *L. zonatus*. We examined eggs and second instar nymphs for evidence of vertical transmission of *Burkholderia*. Similar to several studies on coreoid bugs performed by Kikuchi and colleagues (16, 17, 32), our results show no evidence for vertical transmission of *Burkholderia* from *L. zonatus* mothers to offspring. Diagnostic PCR did not amplify *Burkholderia* from any of 113 eggs or 71 early second instar nymphs. Our experimental results for both *L. zonatus* and *L. phyllopus* also confirm that *Burkholderia* is not vertically transmitted in leaf-footed bugs. Only one *L. zonatus* adult in the aposymbiotic treatment (of 20 tested) was found to carry *Burkholderia*, while in *L. phyllopus*, none of the three aposymbiotic bugs that became adults were *Burkholderia* infected. Given the absence of *Burkholderia* in our PCR survey of eggs and nymphs, the one *Burkholderia*-infected *L. zonatus* adult in the aposymbiotic treatment was more likely to have become infected from laboratory contamination in our experimental arenas than by low rates of vertical transmission.

Role of *Burkholderia* isolate identity in bug development and performance. We asked whether four different symbiotic species of *Burkholderia*, all from the clade most consistently associated with coreoid and lygaeoid bugs (the SBE clade) (Fig. 5), could differentially affect bug performance. We found that symbiont identity influenced the performance of both *L. zonatus* and *L. phyllopus*. The effects of four different isolates on insect development time and fresh weight at adulthood were qualitatively very similar for the two insect species. Specifically, *L. zonatus* developed 12% slower when colonized by isolate Lep1P3 than when colonized by isolates Lep1A1 or TF1N1 (Fig. 6a) ($df = 3$, $\chi^2 = 22.4$, $P < 0.0001$). Upon emergence, *L. zonatus* adults colonized by strain Lep1P3 were 29.9% smaller than insects associated with the three other species (Fig. 6b) ($df = 3$, $\chi^2 = 15.3$, $P = 0.0002$). *Leptoglossus phyllopus* individuals developed 21%

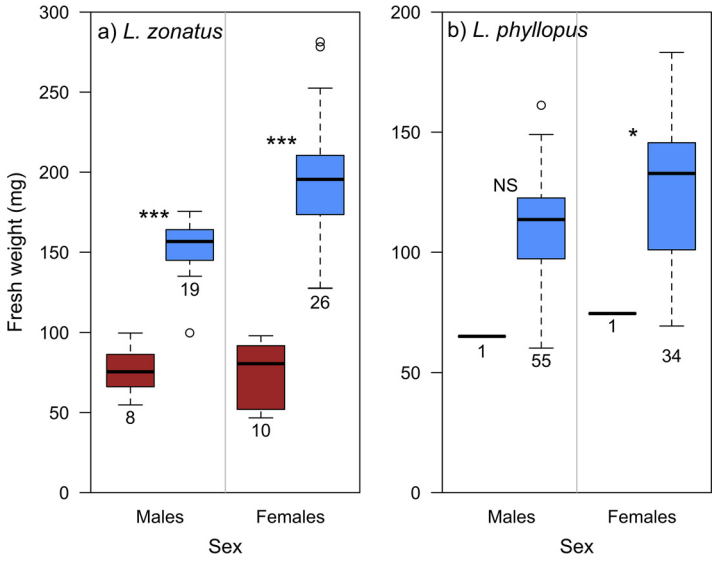

FIG 3 Effect of symbiont status (aposymbiotic [red] versus symbiotic [blue]) and sex on fresh weight of *Leptoglossus* at adulthood. Boxplots show the raw data. Weights were analyzed in mixed regression models (separate models for each species) with cage as a random effect and symbiont colonization status and sex as fixed effects. Sample sizes are reported underneath each boxplot. (One additional aposymbiotic *L. phyllopus* was omitted from this analysis because it was not weighed at eclosion.) Sex and symbiont status were both statistically significant in their effects on fresh weights in both models, but in *L. phyllopus*, the difference between aposymbiotic and symbiotic males was not statistically significant, likely due to the low number of aposymbiotic individuals that reached adulthood.

slower when colonized by isolate Lep1P3 than when colonized by any of the other three isolates (Fig. 6c) ($df = 3$, $\chi^2 = 21.2$, $P < 0.0001$), and adults associated with isolate Lep1P3 were 30.1% smaller than those colonized by the three other isolates (Fig. 6d) ($df = 3$, $\chi^2 = 36.7$, $P < 0.0001$). Interestingly, similar to the *L. zonatus* aposymbiotic

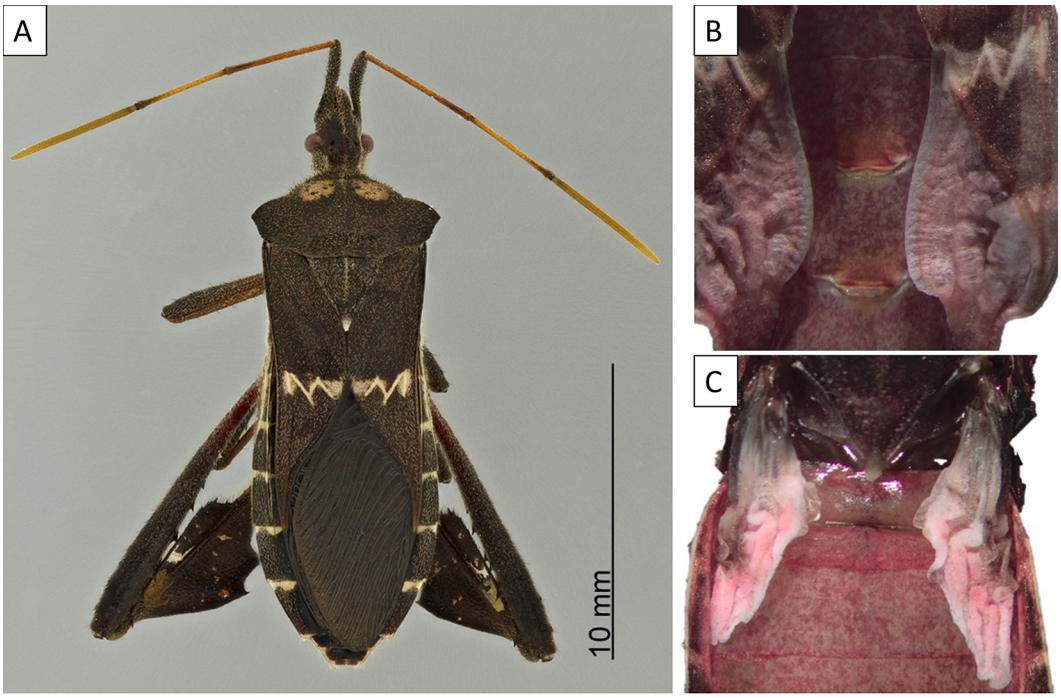

FIG 4 *Leptoglossus zonatus*. (A) Healthy adult female, typical of the symbiotic condition. (B) Detail of an aposymbiotic adult with a front wing deformity—front wings are shriveled and do not meet over the dorsum. (C) Detail of an aposymbiotic adult with a back wing deformity. The front wings have been removed to show the crumpled, unexpanded hind wings. Half of the adults that survived to adulthood in the aposymbiotic treatment (10 of 20) had visible deformities, molting difficulties such as attached fifth instar cuticle, or sex ambiguity.

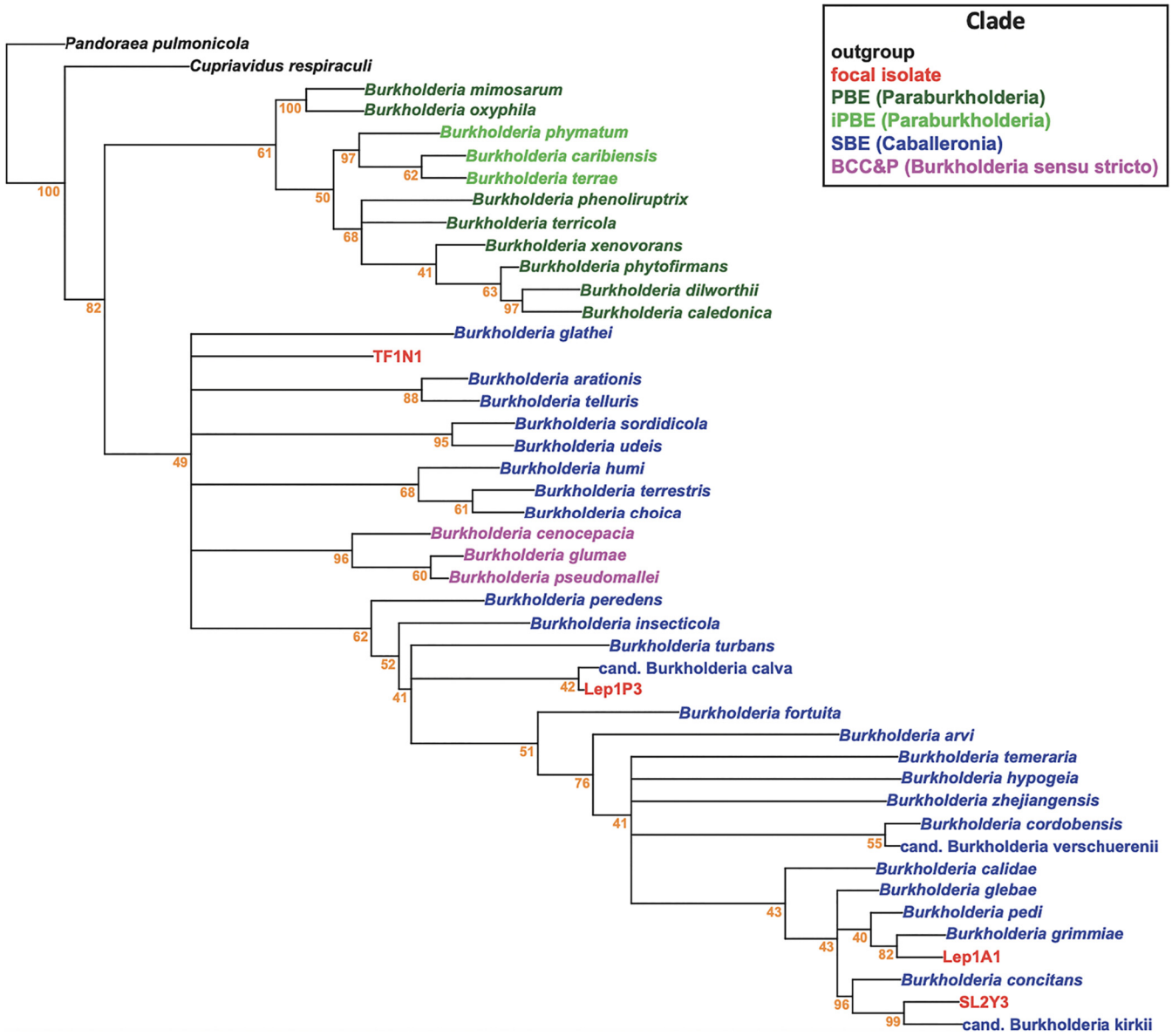

FIG 5 Phylogeny of *Burkholderia* showing placement of the four isolates used in the performance experiments. 16s rRNA sequences of the focal isolates were 1,532 to 1,534 bp in length. Selected *Burkholderia* sequences downloaded from GenBank varied from 1,231 to 1,553 bp in length. The consensus maximum likelihood phylogeny was inferred using RAxML v8.2.11 with the GTR GAMMA model of nucleotide evolution and rooted with the outgroup *Pandoraea pulmonicola*. Node support values were calculated using rapid bootstrapping, and nodes with less than 40% support have been collapsed. Text labels are colored according to membership in several named clades. SBE, stinkbug-associated beneficial and environmental; PBE, plant-associated beneficial and environmental; iPBE, the insect-associated subclade of the PBE group; BCC&P, *B. cepacia* complex and *B. pseudomallei* group. The full data set for the phylogeny is available at Dryad (69), and the isolate 16S rRNA sequences have been submitted to NCBI under the following accession numbers: Lep1A1, OK037557; Lep1P3, OK037558; SL2Y3, OK037559; TF1N1, OK037560.

treatment results, 3 out of the 23 *L. phyllopus* individuals infected with isolate Lep1P3 that reached adulthood had deformed wings or hind legs.

However, both bug species performed better when colonized by Lep1P3 than by no *Burkholderia* at all. For *L. zonatus*, comparisons between the aposymbiotic-symbiotic experiment and the separate four-isolate experiment should be made cautiously and only qualitatively, but only 38% of aposymbiotic nymphs reached adulthood, while 88% of nymphs colonized by Lep1P3 reached adulthood. The aposymbiotic nymphs that survived took 32.8 days, on average, to develop from second instar to adulthood, while those colonized by Lep1P3 took 28.3 days on average (14% faster). Aposymbiotic females averaged 75 mg, and males averaged 76 mg at adulthood, while Lep1P3-colonized females weighed 144 mg (92% more) and males weighed 106 mg (38%

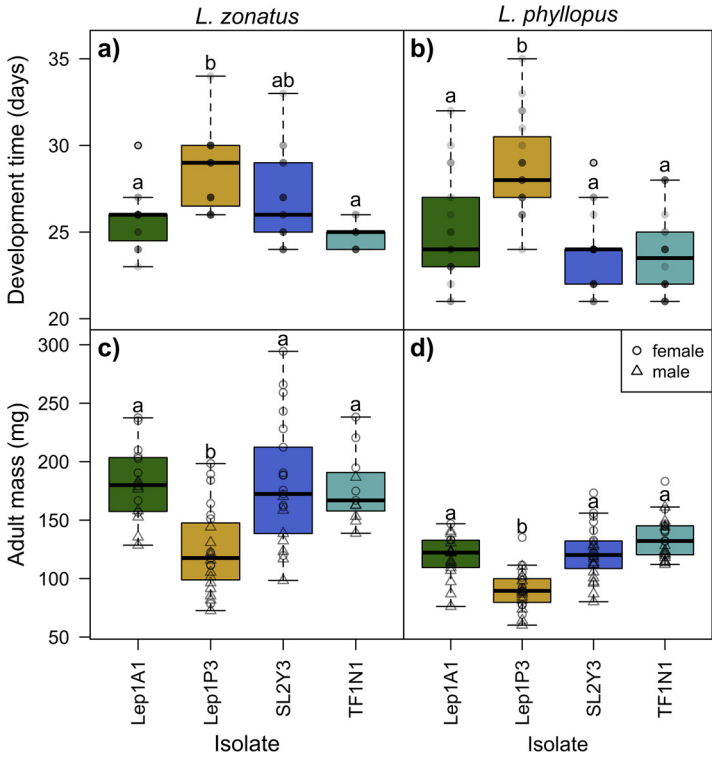

FIG 6 Effects of four different *Burkholderia* isolates on development time and adult fresh weight in two species of *Leptoglossus*. The four isolates were cultured from a laboratory colony of *Leptoglossus zonatus* originally collected in California (Lep1A1 and Lep1P3) or from the stilt bug *Jalysus wickhami* (SL2Y3 and TF1N1, from Arizona and North Carolina, respectively). Development time in days of *L. zonatus* (a) and *L. phyllopus* (b). Adult mass of *L. zonatus* (c) and *L. phyllopus* (d), with data from both males (open triangles clustered on the lower side of each box plot) and females (open circles showing the higher weights of females) plotted on the same figure. Different letters above the boxplots denote statistically significant differences. For both host species, strain Lep1P3 was significantly less beneficial than the other three isolates. Bugs of both species were significantly smaller and took longer to develop when infected with this isolate.

more) at adulthood. We can directly compare aposymbiotic and Lep1P3-colonized *L. phyllopus* individuals because they were examined in the same experiment. Here, after accounting for the random effect of cage, nymphs colonized by Lep1P3 were 3.4 times more likely to survive to adulthood than aposymbiotic nymphs ($df = 1$, $\chi^2 = 19.3$, $P < 0.001$). *Leptoglossus phyllopus* with Lep1P3 developed 43% faster than the few aposymbiotic nymphs that reached adulthood ($df = 1$, $\chi^2 = 17.6$, $P < 0.001$). We were only able to obtain adult masses for two adult aposymbiotic *L. phyllopus*, so the comparison between these individuals and Lep1P3-colonized bugs did not have sufficient statistical power, but after accounting for sex differences and the effect of cage, the average aposymbiotic adult was estimated to weigh only 76% of what the average Lep1P3-colonized adult weighed ($df = 11.6$, $t = 1.6$, $P = 0.13$).

DISCUSSION

When deprived of symbiotic *Burkholderia*, *Leptoglossus* bugs were severely compromised. Symbiotic bugs developed much more rapidly and were four times more likely to survive to adulthood than aposymbiotic bugs. The few aposymbiotic bugs that reached adulthood were approximately half the weight of symbiotic adults and were commonly deformed. Preliminary observations of reproduction among aposymbiotic and symbiotic *L. zonatus* individuals suggest aposymbiotic female bugs do not lay eggs (E. F. Umanzor, unpublished data). While the consequences of being without *Burkholderia* were severe for *L. zonatus*, they were perhaps even greater for *L.*

phyllopus, where only three individuals of a starting cohort of 28 in the aposymbiotic treatment reached adulthood. These results suggest that *Burkholderia* should be considered an obligate associate for both *Leptoglossus* species.

***Burkholderia* likely provides necessary nutrients to *Leptoglossus*.** While we do not yet know exactly what *Burkholderia* provides to *Leptoglossus*, Ohbayashi et al. (21) showed that *Burkholderia insecticola* in *R. pedestris* may contribute to both protein and B vitamin metabolism. This is consistent with the fact that both development time and body size are affected in aposymbiotic *L. zonatus* and *L. phyllopus*. A developmental delay is an expected result of poor nutrition in at least holometabolous insects; larvae grow until they reach a "critical weight" and then prepare for pupation and adulthood (47). Nutrition also influences the critical weight, which is lower in insects fed poor-quality diets (48), allowing insects to eclose as adults at a smaller size.

The cause of the adult cuticle deformities that we observed in aposymbiotic *L. zonatus* is unclear but may relate to their small size or malnourishment. Aposymbiotic *Jalysus wickhami* are not much smaller than symbiotic individuals but are pale and soft as adults (24). It is also possible that the symbiont may play a role in cuticle hardening as has been shown for multiple bug and beetle symbionts (49, 50).

Interestingly, the degree of dependency of bugs on *Burkholderia* appears to vary among host species. The fitness deficits observed in aposymbiotic *Leptoglossus* appear similar in magnitude to other coreids studied (32, 34, 51). These deficits are greater than those observed in the model alydid *R. pedestris*, where aposymbiotic bugs have multiple fitness deficits but can still reproduce (16, 30, 31), or the alydid *Alydus tomentosus*, where aposymbiotic bugs have only slightly higher developmental mortality (23). Similarly, for the berytid *J. wickhami*, aposymbiotic bugs developed more slowly and laid fewer eggs but did still reproduce (24). This raises the question of what causes differences among host species in the magnitude of benefits provided by *Burkholderia*. Possibilities for future investigation include the nutritional quality of the hosts' native diets or physiological differences among the species themselves.

Despite its importance in host development, *Burkholderia* is not vertically transmitted. Our data suggest that vertical transmission of *Burkholderia* does not occur. We found this in an explicit test for vertical transmission in *L. zonatus*, where 113 eggs and 71 early instar nymphs were tested. Furthermore, in experiments with an aposymbiotic treatment, no *L. phyllopus* and just one *L. zonatus* were positive for *Burkholderia* at adulthood, the latter consistent with low levels of laboratory contamination. These results support the multiple studies of Kikuchi and colleagues that show exclusively environmental transmission of *Burkholderia* among members of the sister coreoid families Coreidae and Alydidae (17, 51).

The lack of a vertical transmission route coupled with the critical requirement that nymphs acquire *Burkholderia* for normal growth raises the question of where the often-arboreal nymphs of *Leptoglossus* species, particularly *L. zonatus*, find *Burkholderia* to ingest, and whether *Burkholderia* acquisition is ever limiting. In studies of *R. pedestris*, *B. insecticola* is not excreted, limiting potential sources of the symbiont for horizontal transmission (16). It is possible that this common soil microbe is effectively "everywhere" as the Baas-Becking hypothesis would posit (52) or at least common in dust blown onto plant surfaces or, perhaps, as a resident of plant vascular tissue where small nymphs may encounter it. Plant-mediated transmission of *Burkholderia* is an intriguing possibility and was suggested by Xu et al. (35), although no clear evidence of this transmission route has been shown to date. Alternatively, horizontal transmission from infected older nymphs and adults may occur in *Leptoglossus* through an as-yet undiscovered route. In the coreid *Anasa tristis*, most offspring reared with adults infected with green fluorescent protein (GFP)-labeled SBE *Burkholderia* acquired the GFP-labeled strain, suggesting such indirect vertical transmission may occur in at least one coreid species (51).

Given the severity of the fitness consequences of being aposymbiotic, our results suggest that virtually all adults of these two species that are observed in the field are symbiotic. While some aposymbiotic bugs did survive to adulthood in the protected

laboratory environment, the high mortality, long development time, adult deformities, and small size of aposymbiotic bugs would make these insects highly vulnerable to both biotic and abiotic sources of mortality in nature. This does not mean that every *Leptoglossus* nymph necessarily gains the symbiont in the field. The question of whether symbiont limitation plays a role in the demography of these insects is a compelling topic for future research.

***Burkholderia* species vary consistently in degree of benefit conferred to two congeneric hosts.** Our results raise the possibility that acquisition of *Burkholderia* isolates that confer variable benefits could lead to fitness differences in bugs in the field. Past work has shown that members of the major clades of *Burkholderia sensu lato*—SBE, PBE (the plant beneficial and environmental clade), and BCC&P (the *Burkholderia cepacia* complex and *Burkholderia pseudomallei* clade)—vary both in their ability to associate with bugs and to benefit them (29). Specifically, research on *R. pedestris* shows that SBE and PBE isolates are capable of colonizing the M4 organ, but SBE isolates outcompete PBE isolates when both are present (29). Members of BCC&P cannot colonize the organ and, therefore, cannot provide any benefit to these insects (29). Furthermore, a comparison of squash bug performance when infected with strains of SBE, PBE, or *Cupriavidus* (in the *Burkholderiaceae*) showed that PBE and *Cupriavidus* infections were not as beneficial as were the two SBE species (51), while the two SBE isolates were equivalently beneficial.

We compared benefits conferred by isolates representing four different species (*Burkholderia* [=*Caballeronia*] *grimmiae* and three unnamed species) within the SBE clade. Bugs fed three of the isolates were comparable to field-collected, naturally-infected bugs in size and appearance. In contrast, we observed that bugs colonized by one of the isolates, Lep1P3, took longer to develop and weighed less at adulthood than those colonized by any of the other three isolates. While we did not directly evaluate reproductive success or longevity, longer developmental time may lead to higher rates of mortality from natural enemies (53). Also, smaller size in adult female insects is associated with lower fertility (54), while smaller leaf-footed bug males have reduced success at competing for females (55). It is important to note, however, that bugs infected with the least beneficial isolate still performed appreciably better than aposymbiotic bugs. As poor as Lep1P3 was as a symbiont, it was still much better for the bugs to associate with this isolate than to have no symbiont at all.

More generally, the variation in performance of bugs infected with different *Burkholderia* isolates shows that there are individual risks associated with environmental acquisition of symbionts even if acquiring some symbiont is virtually guaranteed. The risks of inferior symbionts may have population-level consequences if *Burkholderia* isolates vary spatially or temporally with respect to effects on *Leptoglossus* fitness, and isolates that lead to poor host performance are sometimes the most abundant. We might then expect a contraction in population size and a reduction in pest pressure. Conversely, the environmental acquisition of symbionts allows for the possibility of association with isolates that are more beneficial for hosts generally or with isolates that are beneficial in a particular context because of local symbiont adaptation. In this case, we might predict population expansion and greater pest problems. This possibility was most elegantly demonstrated by Kikuchi and colleagues (46) who showed that *R. pedestris* colonized by *Burkholderia* strains from fields with high fenitrothion exposure were themselves more resistant to this common insecticide (46).

Interestingly, although we found variation in the benefits of different *Burkholderia* isolates, both *Leptoglossus* species were almost identical in how they responded to that variation. There was no evidence, for example, that the symbiont strains isolated from *L. zonatus* were better for *L. zonatus* than for *L. phyllopus*, and in fact, the lower-performance symbiont strain (for both hosts) was isolated from *L. zonatus*, while the two isolates derived from the distantly related *J. wickhami* (Berytidae) were similarly beneficial in their effects on *L. zonatus* and *L. phyllopus* as was the second isolate derived from *L. zonatus*. Further, although we cannot make quantitative comparisons between the two species of *Leptoglossus* because they were reared in different labs at

different times, the results were extremely similar for both host species, suggesting a lack of fine-grained specificity at the level of congeneric host species. A lack of strain specificity has been found in other hemipteran-*Burkholderia* associations as well, including in alydids and the berytid *J. wickhami* in North America (23, 24), coreids in Japan and Europe (32), stenocephalids in Europe (33), and a blissid in Japan (56). Geographic structuring of strains has been shown in some studies, however (24, 32), and a clade within SBE *Burkholderia* has been shown to be more often associated with the coreoid family Stenocephalidae (33).

The overlooked role of *Burkholderia* in coreid biology. Several coreid bugs, including species in *Leptoglossus* and *Anasa*, are important pests, and others, including *Narnia*, *Thasus*, and others have been models for the study of male weaponry and sexual selection (55, 57, 58). We now know that coreids must associate with *Burkholderia* for optimal performance, yet the central role of symbiosis in the biology of these insects has not been mentioned in recent publications that focus on their life history and ecology (e.g., see references 59–61). This is despite the groundbreaking research of Kikuchi and colleagues that detailed the taxonomic distribution of lygaeoid- and coreoid-*Burkholderia* associations in 2011 (17) and showed the critical importance of *Burkholderia* for development and survivorship of the coreid, *Coreus marginatus* (32).

At minimum, knowing that *Burkholderia* is required in these insects will help investigators design laboratory rearing systems that are likely to be successful and efficient (i.e., those including soil as a source of the symbiont). Reading older literature with the hindsight provided by recent research, there are hints that rearing leaf-footed bugs without awareness of the obligate symbiosis may be challenging. Vessels et al. (62) attempted laboratory rearing of *Narnia femorata* without soil on cactus and fruits and somewhat cryptically mention that eggs and first instars were reared from adults in the laboratory, but subsequent stages studied were brought in from the field, presumably because laboratory-reared later instars were aposymbiotic and did not survive, while field collected nymphs were symbiotic. Similarly, low survivorship of *L. zonatus* is reported in a study that attempted an artificial rearing system where no soil was provided (63).

More importantly, understanding the role of the symbiont in the life history of these insects could improve our insight into them generally. For example, knowing that the timing of acquisition of the symbiont is likely to influence performance (27) could explain a finding that the fastest developing individuals in a group of siblings of *N. femorata* are also the biggest in adulthood (64). Lastly, our results suggest that population dynamics of *Leptoglossus* pests may be influenced by symbiont strain identity and, if symbiont acquisition is ever limiting, by access to the symbiont itself.

MATERIALS AND METHODS

***Leptoglossus zonatus* culture.** *Leptoglossus zonatus* adults were collected in a pomegranate orchard near Mettler, Kern County, CA, USA in October 2017 and established in the laboratory at the University of Arizona (Tucson, AZ, USA) in large screened plexiglass cages (30 by 30 by 30 cm) in a walk-in incubator set at 27°C, 16 h light/8 h dark. The cages contained whole cowpea plants (*Vigna unguiculata*) potted in Promix LP 15 potting mix in 15-cm pots and raw Spanish peanuts glued with a glue gun in arrays to index cards. *Leptoglossus* are seed and fruit feeders and may also imbibe xylem sap (36, 40).

***Leptoglossus phyllopus* culture.** *Leptoglossus phyllopus* adults were collected from flowering weeds in an urban lot in Arlington, Texas in September 2019. A laboratory colony was established at the University of Texas at Arlington (Arlington, TX, USA) in large mesh cages kept in the laboratory at room temperature (~24°C) with a 16 h light/8 h dark cycle. Cages contained potted cowpea plants and raw Spanish peanuts as described for *L. zonatus*.

Vertical transmission of *Burkholderia* in *L. zonatus*. While research on other coreids suggested *Burkholderia* is not vertically transmitted in this family (17, 33, 34), we assessed the possibility of vertical transmission of *Burkholderia* in arboreal *L. zonatus*. We placed 10 adult pairs in each of two large jar cages (one gallon plastic jars with ventilated lids) and provided raw peanuts and distilled water containing 0.05% L-ascorbic acid. *Leptoglossus* females lay their eggs in linear clutches of approximately 30 eggs, often along stems of plants or mid-ribs of leaves. We collected 113 eggs from approximately 11 clutches (9 to 11 eggs per clutch) and placed each egg in a 0.5-ml tube. Eggs were frozen at −20°C. To test for the presence of *Burkholderia* in or on the eggs, we performed DNA extraction and diagnostic PCR (methods described in the section "Diagnostic PCR for infection status") with positive and negative *Burkholderia* controls. Other eggs were placed in petri dishes with peanuts and water vials (with 0.05%

ascorbic acid). Nymphs were allowed to hatch and develop until the beginning of the second instar and then were collected as described above. The DNA of 71 whole nymphs was extracted and surveyed via diagnostic PCR for *Burkholderia*.

The role of the symbiont in development and performance in *Leptoglossus*. For the two *Leptoglossus* species—*L. zonatus* studied in a laboratory in Tucson, AZ, and *L. phyllopus* in Arlington, TX—experiments were set up slightly differently. For *L. zonatus*, two experiments were performed. In the first, the development and performance of symbiotic and aposymbiotic nymphs was compared. In the second, nymphs were fed with one of four *Burkholderia* isolates and their performance measured. For *L. phyllopus*, both comparisons were made in one experiment with the same four *Burkholderia* isolate treatments and an additional aposymbiotic treatment. For this reason, we first describe the experiments performed with *L. zonatus* and then simply highlight the slight differences in methods used for the *L. phyllopus* experiment.

***L. zonatus:* aposymbiotic versus symbiotic comparison.** A culture cage was set up with approximately 15 adult pairs and inspected each day for newly laid eggs. Eggs were removed from the culture cage and placed in petri dishes for observation. When egg hatch began, water vials were added to the petri dishes. First instar nymphs were then collected and distributed among screened plexiglass boxes (11.33 cm by 11.33 cm by 4 cm [see Fig. S1 in the supplemental material], hereafter referred to as cages for clarity) for the start of the experiment. Before use in the experiment, each cage was thoroughly washed, soaked in a 0.1% sodium hypochlorite solution, and then rinsed with deionized water and dried. Each cage was provided with a small cowpea seedling that had been removed from soil, its roots and leaves thoroughly rinsed and the plant placed in a floral water vial with cool, not yet gelled 0.4% water agar. Cages were also provided with a 7.5-cm by 1.5-cm cotton-stoppered vial filled with deionized water. Water for both plant and water vial were refilled as necessary during the experiment. Four raw peanuts were added to the side of the cage, each one secured with Loctite Fun-Tak mounting putty (Fig. S1).

After seven first instar nymphs were assigned to each cage, cage replicates were assigned to either aposymbiotic (without *Burkholderia*) or symbiotic treatments. When nymphs graduated to the second instar, plants and water vials were removed from the cages to dehydrate nymphs for 24 h. At the same time, a glycerol stock of *Burkholderia* ("Lep1A1") was used to inoculate 12- by 75-mm culture tubes containing 1 ml of yeast-glucose (YG) medium, and cultures were grown overnight at room temperature. The broth was transferred to 1.5-ml centrifuge tubes, which were then centrifuged for 5 min at 5,000 $\times$ *g*, the supernatant removed from the pellet, and the pellet resuspended in 1.5 ml water. Symbiotic treatment cages then each received a cotton-stoppered 1.2-ml tube containing the *Burkholderia* suspension. Aposymbiotic cages received a cotton-stoppered vial of water. After 24 h, the vials in the symbiotic treatment cages were replaced with a fresh *Burkholderia* suspension for an additional 24 h (48 h total); aposymbiotic cages received a fresh vial of water. After the 48-h exposure, all vials containing *Burkholderia* were removed, and water vials and plants were replaced.

Ten cages per treatment were started, but nymphs in a few cages suffered high rates of mortality of first and second instar nymphs prior to any symbiont treatment. When cages with high rates of early instar mortality were excluded from the experiment, there were seven replicates per treatment.

Each day, the number of dead nymphs and live nymphs in each stage was recorded. Dead nymphs were removed and discarded. When the surviving bugs eclosed to adulthood, they were sexed and weighed, and then dissected. The M4 region of the gut was removed for subsequent diagnostic PCR to determine infection status (see Fig. S2 in the supplemental material; see the section "Diagnostic PCR for infection status" below).

Before analysis, any adult that did not conform with the expected infection status of the treatment (e.g., aposymbiotic or symbiotic) was removed from further analysis. This included one *L. zonatus* adult that tested positive in the aposymbiotic treatment (out of 20 total aposymbiotic adults) and two adults that were negative in the symbiotic treatment (out of 45 total symbiotic adults). The PCR product from the putatively positive aposymbiotic individual was sequenced, and it was found to be a *Burkholderia* that did not match the strain used in the infection process, suggesting a low level of contamination from the laboratory environment.

***L. zonatus*: role of *Burkholderia* strain identity in bug development and performance.** To determine whether *Burkholderia* isolates were equivalent in their influence on *L. zonatus* development and performance, we compared the development of bugs inoculated with four different isolates of *Burkholderia* (see "Isolation of symbiotic *Burkholderia* strains" below for details). Eggs were placed in petri dishes for observation until hatch, and then seven first instar nymphs were added to each cage. When nymphs reached the second instar, we followed the same procedure of dehydrating nymphs, culturing the symbiont, and providing *Burkholderia* suspensions to the symbiotic treatments (and water to the aposymbiotic treatments) as described above, with the one difference that fresh cell suspensions were placed in each cage daily for 3 days instead of 2 days. Four cages of seven nymphs were started for each of the *Burkholderia* isolates. When insects reached adulthood, their sex and fresh weight was recorded.

***L. phyllopus*: role of symbiont presence and identity in development and performance.** We examined the role of *Burkholderia* (presence and isolate identity) in *L. phyllopus* in one experiment that was very similar to those described for *L. zonatus*, with some small differences. We put 5 first instar nymphs in each cage. We assigned six cages to each of five treatments as follows: aposymbiotic and each of four *Burkholderia* isolates, described below ("Isolation of symbiotic *Burkholderia* strains"). When nymphs reached the second instar, we removed the water and plant to dehydrate them as described above. Then, fresh *Burkholderia* cell suspensions were placed in the cage in 1.5-ml vials every 24 h for 3 days. The optical density (OD) of the cell suspension was normalized to 0.2. By plating a dilution series

on YG agar and counting CFU, we determined that this correlated to a live cell density of approximately 3.5×10^5 CFU per μL. High early second instar mortality in one of the cages in which nymphs were exposed to *Burkholderia* isolate TF1N1 resulted in a total of five cages for this treatment. Six replicates remained for the remaining four treatments. Fresh weights were recorded for each adult that emerged. Adults were then frozen at −80°C until DNA extraction was performed.

Out of 88 total individuals tested for the presence of *Burkholderia*, two *L. phyllopus*—one exposed to strain TF1N1 and one exposed to strain SL2Y3—tested negative. Data from these individuals were discarded prior to analysis.

Isolation of symbiotic *Burkholderia* strains. For the strain comparison experiments, both *L. zonatus* and *L. phyllopus* were exposed to one of a set of four *Burkholderia* isolates. Two isolates, Lep1A1 and Lep1P3, were cultured from a laboratory colony of *L. zonatus* collected in California and established at the University of Arizona. The two additional isolates, SL2Y3 and TF1N1, were derived from wild-caught stilt bugs, *Jalysus wickhami* (Berytidae) collected in Arizona and North Carolina, respectively. Bugs in the lygaeoid family Berytidae are also known to acquire *Burkholderia* from the environment (17), and *J. wickhami* receives a clear benefit from housing *Burkholderia* (24).

To isolate the symbiont, individual insects were sacrificed by submerging them in 95% ethanol for 2 min. Each insect was dissected in sterile saline, and the M4 gut region was carefully excised. We rinsed the M4 region in sterile water to remove any bacteria on the surface of the organ, then cultured it whole in YG (yeast-glucose) broth at room temperature for 24 to 48 h, shaking at 270 rpm. This organ culture step increases the success of isolations, presumably by allowing the *Burkholderia* to acclimate more gradually to a free-living mode of life (65). The M4 was then removed from the broth, homogenized, and plated on YG agar. Individual colonies were streaked to isolation. DNA was extracted from overnight cultures, and the genomes of our four isolates were sequenced by P. Stillson and A. Ravenscraft (unpublished data). Briefly, DNA was sequenced on Oxford Nanopore MinION and Illumina HiSeq 4000 platforms, and a hybrid assembly was performed. Full-length ribosomal 16S sequences were extracted from the genomes using ContEst16S (66) and aligned to reference 16S rRNA sequences downloaded from NCBI GenBank, and these sequences were used to prepare a phylogeny (Fig. 5).

More insight into the identity of the four focal isolates is provided in the genome study (P. Stillson and A. Ravenscraft, unpublished data). Based on average nucleotide identity (ANI), that study identified Lep1A1 as *Caballeronia grimmiae* (ANI, 97.0%). The closest named relative of TF1N1, Lep1P3, and SL2Y3 was *Caballeronia concitans*, though these three isolates are not *C. concitans*, nor are they the same species as each other (all pairwise ANIs were $<$86.2%, where an ANI above 95% indicates that two genomes derive from the same bacterial species) (67). We note that *Caballeronia concitans* does not appear to be the closest relative of Lep1P3 and TF1N1 in Fig. 5 because this phylogeny is based only on 16S rRNA gene sequences, not whole-genome ANI comparisons. Also, the taxonomy of *Burkholderia sensu lato* is in flux, and *Caballeronia* is a new genus name that has been attached to insect-associated species that were previously placed in *Burkholderia sensu lato* (25). All isolates used in our experiments are members of the *Burkholderia* stinkbug beneficial and environmental (SBE) clade of Kikuchi and colleagues (56).

Diagnostic PCR for infection status. To diagnose the *Burkholderia* infection status of *L. zonatus*, a small portion of the M4 region of an adult *L. zonatus* was homogenized with the tip of a pipette in an 8-μL drop of proteinase K on the side of an Eppendorf tube and then pushed into a Chelex mixture (10% by weight sterile Chelex beads [Sigma-Aldrich] in 100 μL of sterile water). The tubes were incubated at 37°C for at least 1 h, then at 96°C for 8 min, and then kept at −20°C until used in PCR. For *L. phyllopus*, the entire abdomen was removed, homogenized in 200 μL phosphate-buffered saline (PBS) via bead beating, and the DNA extracted with the Qiagen DNeasy blood and tissue kit.

The presence of *Burkholderia* was determined using specific primers for *Burkholderia* 16S rDNA described above (68). For both species, each 20-μL PCR mixture contained 0.4 μM each primer, 240 μM each deoxynucleoside triphosphate (dNTP), 1.2 U OneTaq (New England Biolabs), 1$\times$ OneTaq standard reaction buffer, and 1 μL DNA extract. In the thermocycler, reactions were denatured at 95°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at 55°C for 1 min, and extension at 68°C for 2 min, with a final extension of 68°C for 6 min. PCR products were visualized on an agarose gel with *Burkholderia*-positive and -negative controls and Sybr green DNA dye mixed into the products. For a subset of samples, the product was confirmed as *Burkholderia* by DNA sequencing at the UA Genetics Core.

Analysis. All analyses were performed separately for each insect species. To estimate the probability of survival with and without the symbiont, we performed a mixed logistic regression with symbiont colonization status as a fixed effect, controlling for cage as a random effect. To measure the effect of symbiont presence on instar-specific development time, we performed a linear mixed effects regression with cage as a random effect and symbiont colonization status and instar reached as fixed effects. To measure the effect of symbiont presence on fresh weight at adulthood, we used a linear mixed effects model with cage as a random effect and symbiont colonization status and sex as fixed effects. To test for differences in development time and fresh mass at adulthood between insects colonized with four different symbiont strains, we performed a linear mixed effects regression similar to those described above, except that we replaced the term for symbiont infection status (aposymbiotic versus symbiotic) with a term for symbiont isolate identity (Lep1A1, Lep1P3, TF1N1, or SL2Y3).

Data availability. All data from this study has been deposited in Dryad data repository (69). Isolate 16S rRNA sequences were additionally deposited in NCBI under the following accession numbers: Lep1A1, OK037557; Lep1P3, OK037558; SL2Y3, OK037559; TF1N1, OK037560.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 2 MB.

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