Report

Current Biology

# Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism

## Highlights

- Choice assays show *A. tristis* nymphs are highly attracted to symbiont-rich feces
- *A. tristis* nymphs use flush feeding to acquire symbionts from fecal matter
- Nymphs are only attracted to conspecific feces, reinforcing host-microbe associations

## Authors

Scott M. Villa, Jason Z. Chen, Zeeyong Kwong, Alice Acosta, Nicole M. Vega, Nicole M. Gerardo

## Correspondence

scott.villa@gmail.com

## In brief

Villa et al. demonstrate how an agricultural pest reliably transmits their beneficial microbial symbionts despite having to acquire them from the environment every generation. Real-time tracking of fluorescently tagged symbionts shows that symbiont-free nymphs use species-specific homing and feeding behaviors to ingest symbionts from adult feces.

Villa et al., 2023, Current Biology *33*, 1–9
July 10, 2023 
https://doi.org/10.1016/j.cub.2023.05.062

CellPress

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062

Current Biology

CellPress

Report

# Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism

Scott M. Villa,[1,2,3,4,7,*] Jason Z. Chen,[1,3,5] Zeeyong Kwong,[1] Alice Acosta,[1] Nicole M. Vega,[1] and Nicole M. Gerardo[1,6]
[1]Department of Biology, Emory University, 1510 Clifton Rd., Atlanta, GA 30322, USA
[2]Department of Biology, Davidson College, 209 Ridge Rd., Davidson, NC 28035, USA
[3]These authors contributed equally
[4]Twitter: @ScottVilla
[5]Twitter: @thyreodon
[6]Twitter: @NicoleGerardo
[7]Lead contact
*Correspondence: scott.villa@gmail.com
https://doi.org/10.1016/j.cub.2023.05.062

## SUMMARY

Understanding how horizontally transmitted mutualisms are maintained is a major focus of symbiosis research.[1–4] Unlike vertical transmission, hosts that rely on horizontal transmission produce symbiont-free offspring that must find and acquire their beneficial microbes from the environment. This transmission strategy is inherently risky since hosts may not obtain the right symbiont every generation. Despite these potential costs, horizontal transmission underlies stable mutualisms involving a large diversity of both plants and animals.[5–9] One largely unexplored way horizontal transmission is maintained is for hosts to evolve sophisticated mechanisms to consistently find and acquire specific symbionts from the environment. Here, we examine this possibility in the squash bug *Anasa tristis*, an insect pest that requires bacterial symbionts in the genus *Caballeronia*[10] for survival and development.[11] We conduct a series of behavioral and transmission experiments that track strain-level transmission *in vivo* among individuals in real-time. We demonstrate that nymphs can accurately find feces from adult bugs in both the presence and absence of those adults. Once nymphs locate the feces, they deploy feeding behavior that results in nearly perfect symbiont acquisition success. We further demonstrate that nymphs can locate and feed on isolated, cultured symbionts in the absence of feces. Finally, we show this acquisition behavior is highly host specific. Taken together, our data describe not only the evolution of a reliable horizontal transmission strategy, but also a potential mechanism that drives patterns of species-specific microbial communities among closely related, sympatric host species.

## RESULTS AND DISCUSSION

We aim to elucidate how host behavior is critical for maintaining specificity in a horizontally transmitted mutualism. We leverage a tractable system consisting of the widespread agricultural pest, *Anasa tristis* DeGeer (Heteroptera: Coreidae), and its beneficial microbial symbiont *Caballeronia* (formerly classified in *Burkholderia sensu lato*)[10,12–15] (Figure 1). *A. tristis* is a squash bug that relies on *Caballeronia* for growth, development, and survival.[11,16] *Caballeronia* is culturable and genetically manipulable, which allows the introduction of stable green (GFP) and red fluorescent protein (RFP) expression (Figure 1D) into different *Caballeronia* strains to facilitate rapid confirmation of strain-specific patterns of colonization in live insects.

The obligate dependence of *A. tristis* on *Caballeronia* should favor selection for direct, vertical symbiont transmission from parent to offspring,[17–20] a strategy employed by many other insects.[21–27] However, *A. tristis* instead relies on horizontal transmission, where adults produce symbiont-free offspring that must acquire their mutualistic bacteria from the environment.[11,12] This strategy is inherently risky, as some nymphs may not find the symbionts they need or may accidently acquire a less beneficial strain.[28] Indeed, recent data now suggest *A. tristis* symbionts are much rarer in the environment than previously realized (J. Garcia, personal communication), making it even more puzzling that they would rely on environmental transmission. Despite these risks, *A. tristis* nymphs consistently obtain *Caballeronia* every generation both in the lab and the field,[11] suggesting they have somehow evolved robust pathways to ensure transmission.

Previous experiments with squash bugs have reported a high frequency of symbiont transmission across generations.[11] Efforts to establish how symbionts are passed in this system have ruled out egg surface contamination as a viable transmission route. Thus, it was assumed that symbiont-free nymphs randomly pick up *Caballeronia* through incidental contact with soil or feeding on plants,[11] as reported for related insects.[5,29]

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062




**Figure 1. The tractability of the squash bug-*Caballeronia* system**

Strains of *Caballeronia* symbionts can be tagged with fluorescent proteins and experimentally fed to lab-reared *A. tristis*.

(A and B) (A) Inoculated adults, which defecate on host plants or surrounding soil, (B) deposit large quantities of fluorescently tagged symbionts in their feces.

(C) By harvesting this excrement, we can expose symbiont-free nymphs to different quantities and strains of *Caballeronia*.

(D) A second instar nymph with GFP-tagged symbionts (left), and two early third instar nymphs with RFP-tagged symbionts (middle; red recolored as magenta for accessibility) or no symbionts (right), illustrate that fluorescent bacteria established in the host will glow through the abdominal cuticle, providing non-destructive confirmation of symbiont establishment. This insect-microbe system provides the unique ability to not only track strain-level transmission *in vivo* among *A. tristis* in real-time, but also provides a way to directly link symbiont availability, host behavior, and transmission success.

See also Video S1.

Here, we demonstrate that *A. tristis* nymphs instead use directed homing behavior to actively seek out their symbionts from adult feces. When adults defecate, nymphs appear to flock to the feces and deploy behavior consistent with coprophagy (Video S1). Moreover, nymphs appear to sense fecal spots from a distance and move directly toward them when available (Video S2). Because food was readily available to nymphs during these initial observations, it is unlikely this response is due to starvation or a misdirected attraction to food-based cues. Given that *A. tristis* adults frequently co-occur with nymphs in natural populations, the ability to home in on feces as a source of *Caballeronia* would be highly beneficial for these nymphs, as it could greatly increase the likelihood of successful transmission.

We therefore conducted a series of choice assays to put *A. tristis*' symbiont finding capabilities to the test (Figure 2A). We filmed groups of symbiont-free nymphs placed in arenas with different combinations of attractants (Figure 2B). In the control arenas, when both choices were PBS solution (i.e., no symbionts), nymphs showed no significant bias to either choice (Figure 2C; paired t test; n = 31; df = 30; t = 0.29; P = 0.77). In arenas where one of the choices was instead a single fecal spot with *Caballeronia*, nymphs displayed extreme bias toward the fecal spot (Figure 2D; n = 17; df = 16; t = −5.38; P < 0.0001). Strikingly, of the 229 visits observed among these trials, 226 (98.7%) were directed toward the *Caballeronia*-positive feces. We followed up on these results with a series of experiments to decouple attraction to the feces from the symbiont itself. We presented nymphs with a choice between PBS and autoclaved feces (Figure 2E). Autoclaving the feces destroys any live symbionts, which would indicate that fecal attractiveness is not reliant on ongoing symbiont metabolic activity. Nymphs in these trials still displayed significant bias toward the *Caballeronia*-negative feces (n = 20, df = 19, t = −5.41, P < 0.0001). Surprisingly, nymphs also displayed significant visitation bias toward just the symbiont in PBS solution (Figure 2F; n = 36, df = 35, t = −2.39, P = 0.02). These results indicate that *A. tristis* nymphs are strongly attracted to both fecal matter and symbionts, and that this attraction is a behavioral trait whose adaptive function is fundamental for symbiont acquisition.

We next tested the mechanisms nymphs use to detect feces and symbionts in the environment. Like other heteropterans,[30,31] including the closely related alydids,[32,33] squash bugs primarily use their antennae to detect contact or volatile chemicals[34] associated with food[33,35–37] or conspecific pheromones.[30,35,38–42] It is therefore likely that nymphs also rely on olfactory cues to navigate toward both the symbiont and fecal matter. We repeated the feces-only and symbiont-only choice assays described above, but this time we ablated the distal flagellomere (segment IV) of the nymphs' antennae. The high density of sensilla on the distal flagellomere (Figure S1), which is observed across Heteroptera,[30–33] suggested its removal might impede detection of olfactory cues from the excrement and symbiont. We found that though nymphs with ablated antennae showed diminished, albeit still significant, bias toward the autoclaved feces (Figure 2F; n = 6, df = 5, t = −3.24, P = 0.02), they completely lost preference for the symbiont by itself (Figure 2H; n = 12, df = 11, t = 0.70, P = 0.49). These results suggest that nymphs rely solely on olfaction to find the symbionts themselves but might integrate other cues, likely visual or social, to find feces. Although it is unknown how frequently nymphs acquire symbionts that are not encapsulated in feces, our choice assays reveal behaviors unlike any previously reported in this group of pests[43,44] and show how *A. tristis* use fecal matter to maximize their chances of finding their symbiont in the environment. Taken

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062



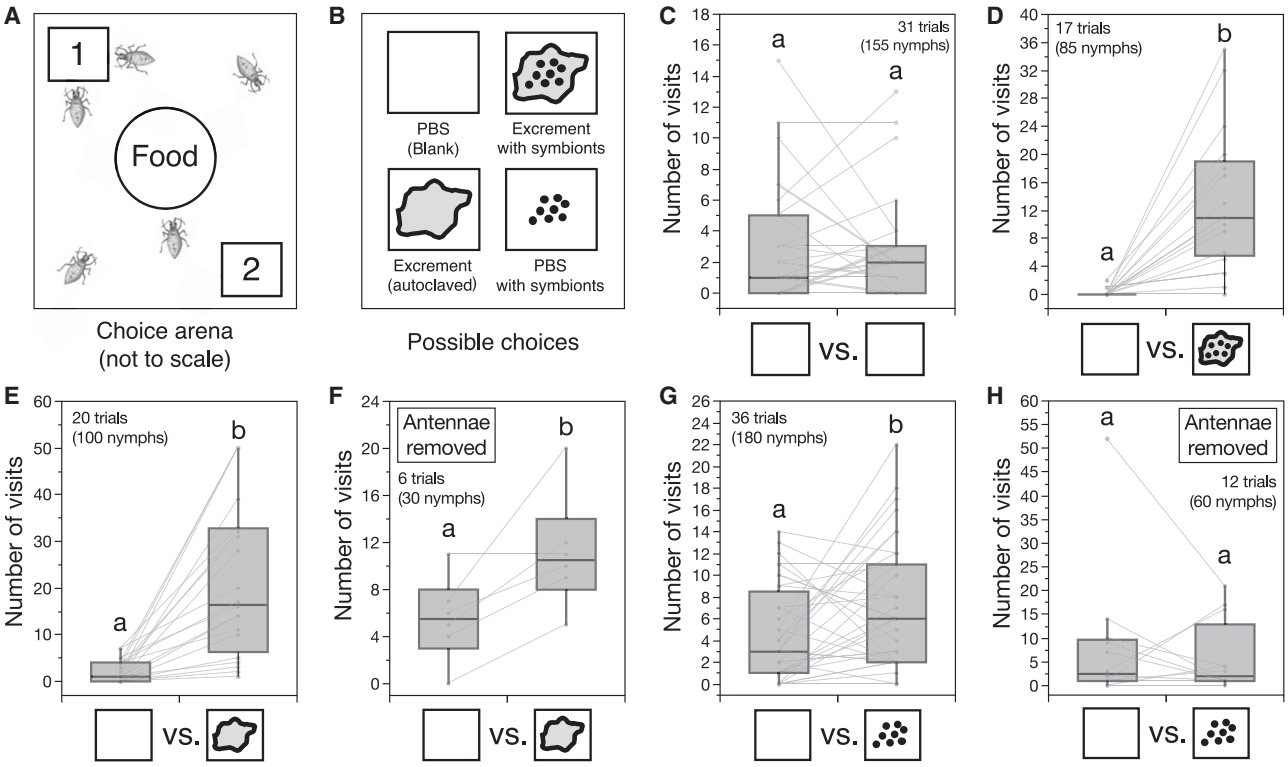


**Figure 2. Influence of symbiont availability on nymph behavior**

(A) *A. tristis* nymphs were placed in a series of choice trials. Each trial consisted of five symbiont-free nymphs placed in an arena with a slice of squash (food source) and two choices of attractants (boxes 1 and 2).

(B) Each choice contained one of four attractants: (1) a pure PBS solution (i.e., a blank); (2) adult excrement with live, GFP-tagged symbionts; (3) autoclaved adult excrement void of live symbionts; or (4) PBS solution with live, GFP-tagged symbionts.

(C–H) Nymphs were placed in trials with different combinations of attractants and recorded continuously with high-definition cameras. Each gray line represents the total number of visits by all five nymphs to each choice in a given trial. In two scenarios (F and H), the distal segment of both antennae for each nymph was removed prior to the start of trials. Different letters indicate a significant ($P < 0.05$) bias between choices.

See also Figure S1 and Videos S1 and S2.

together, these results are consistent with accumulating evidence that environmental microbes engage in chemical dialogues with animal hosts that modulate host behavior and effect their own transmission.[45–49]

The fecal transmission behaviors of *A. tristis* are similar to those in related insects, such as stinkbugs, that transmit specific microbial partners to their offspring via frass or other anal secretions.[50–58] In these taxa, however, symbionts are deposited in association with eggs, rendering transmission effectively vertical. Instead, our data counter many comparable studies of horizontally transmitted mutualisms that suggest hosts randomly find their beneficial partners (but see references Kim et al.[32,33]). For example, the alydid bean bug *Riptortus pedestris*, which also hosts *Caballeronia* symbionts, does not exhibit directed searching behavior during symbiont acquisition, either for symbiont cultures[44] or feces.[59] Instead, *R. pedestris* appears to acquire symbionts opportunistically from the host plant rhizosphere[59] and employs sophisticated mechanisms that enrich for *Caballeronia* within the midgut itself.[60–63] This exclusive reliance on a physiological post-acquisition mechanism (i.e., partner choice) has been well characterized in a diversity of insect[59,64–66] and non-insect mutualisms, including those in the well-studied legume-rhizobial nitrogen-fixing symbiosis[67–71] and the squid-*Vibrio* luminescent symbiosis.[8,72–74] However, these post-acquisition filtering strategies are still predicated on opportunistic encounters with the right symbiont. Such mechanisms are theoretically problematic when host and microbial community compositions are patchy.[3] Moreover, indiscriminate uptake of microbes from the environment leaves the host vulnerable to parasites, pathogens, and cheaters.[28,62,75] By implementing sensitive pre-acquisition detection behaviors, *A. tristis* nymphs can mitigate these risks by homing in on specific sources of their primary symbiont. Such a strategy should theoretically reduce the time, energy, and risk associated with combing the environment for opportunistic encounters.[28]

Because *A. tristis* nymphs presumably incur substantial energetic cost and predation risk to forage for feces, a complementary mechanism should evolve to halt this behavior upon successful symbiont uptake. Indeed, preliminary observations indicated that symbiont-positive nymphs spend most of their time feeding and very little time wandering around their enclosures. Nymphs that were deprived of symbionts displayed the opposite behavior, where a vast majority of their time was spent walking around and very little time feeding (Video S3). We

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062



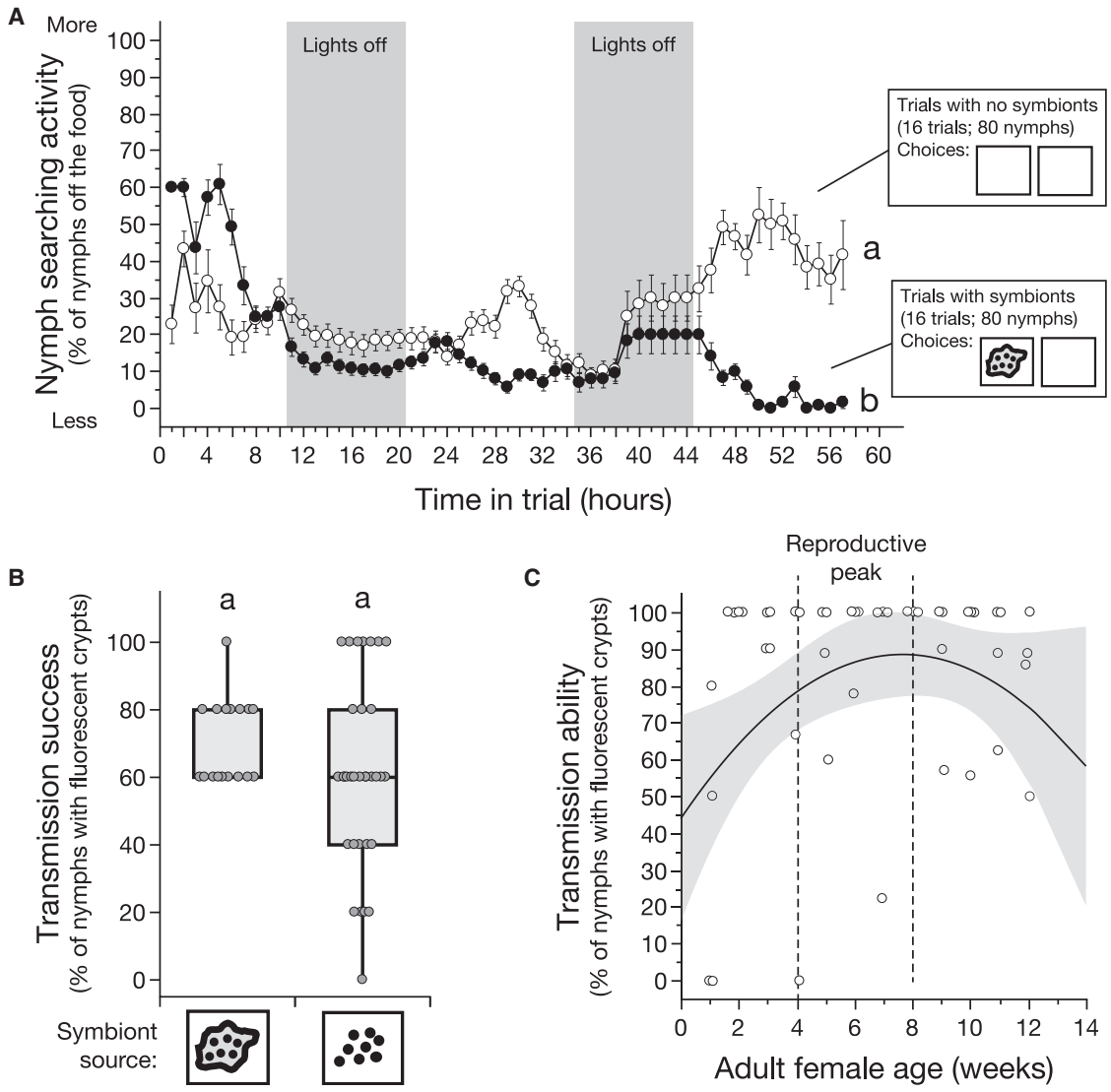


**Figure 3. Symbiont transmission dynamics**

(A) Nymph movement was quantified in a subset of choice trials from Figures 2C and 2D. Searching behavior (mean ± se) was assessed every 10 mins in boxes that had no symbionts (i.e., both choices were blanks, Figure 2C) and those that had excrement with symbionts (Figure 2D). Different letters indicate significant differences in overall searching activity between the two types of trials.

(B) In two types of choice trials where symbionts were available (Figures 2D and 2F), all nymphs were removed from arenas and examined for GFP fluorescence. Transmission was considered successful if nymphs had fluorescent bacteria in their midgut (see Figure 1D for example). Each point represents the percentage of nymphs (out of 5) that were positive for symbionts for a given trial. Different letters indicate significant differences ($P < 0.05$).

(C) Ability of adult females to transmit symbionts throughout their lifetime. Groups of four adult females (n = 4 groups) were exposed to a new set of 10 symbiont-free nymphs every week for 12 weeks, which is the approximate lifespan for *A. tristis*. The percentage of nymphs that acquired symbionts was assessed each week (white points). Reproductive peak of females lies within the dashed lines and indicates when females are most fecund (from Villa et al.[76]).

See also Data S1 and Videos S3 and S4.

therefore revisited the videos of our choice assays, rewatching a subset of arenas to determine if symbiont availability influenced levels of *A. tristis* activity. We re-analyzed two types of choice assays: (1) arenas where both choices were PBS (i.e., no symbionts available to nymphs; Figure 2C) and (2) arenas where one choice was PBS and the other was feces with symbionts (i.e., symbionts were available to nymphs; Figure 2D). We found that nymphs without access to symbionts displayed significantly more searching behavior than those with symbionts (Figure 3A; Data S1A; GLMM, $P < 0.0001$). Amazingly, this behavioral difference appears more exaggerated the longer nymphs were deprived of *Caballeronia*. At the beginning of the trials, nymphs in both treatments spent similar amounts of time searching the arena. However, following this initial acclimation period, most nymphs in the trials that contained symbionts successfully acquired *Caballeronia*, ceased wandering, and consistently stayed on the food for the remainder of the experiment. Nymphs without access to symbionts displayed the opposite behavioral patterns

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062



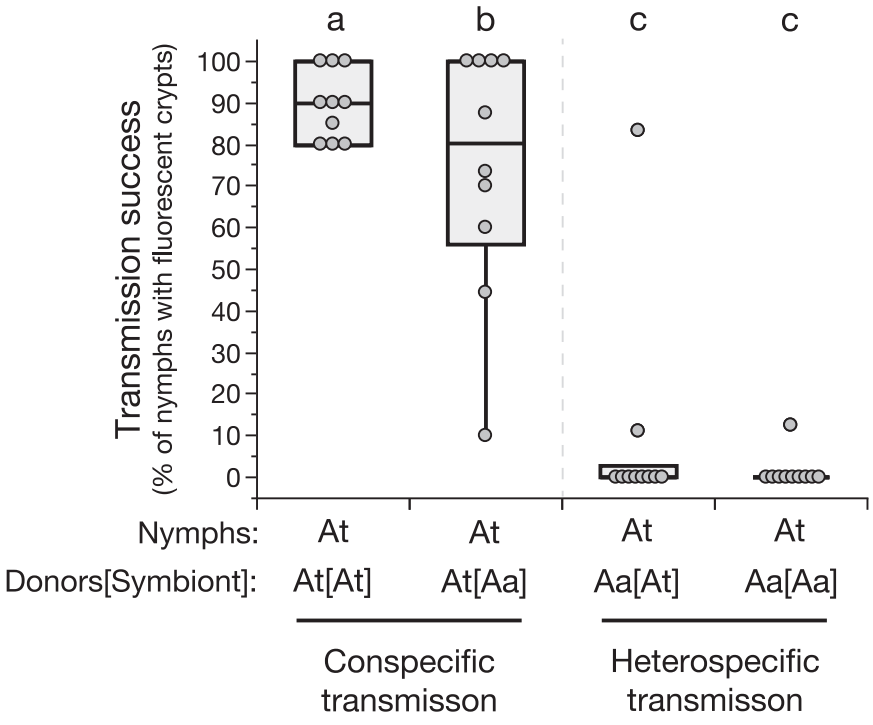


**Figure 4. Transmission specificity**

Groups of symbiont-free *A. tristis* (At) nymphs were placed on plants with either: (1) *A. tristis* adults harboring *A. tristis* symbiont strains (At[At]; n = 10 groups); (2) *A. tristis* adults harboring *Anasa andresii* (Aa) symbiont strains (At[Aa]; n = 10 groups); (3) *A. andresii* adults harboring *A. tristis* symbiont strains (Aa[At]; n = 10 groups); or (4) *A. andresii* adults harboring *A. andresii* symbiont strains (Aa[Aa]; n = 10 groups). Letters indicate significant differences (ANOVA with Tukey post-hoc tests, $P < 0.05$).

See also Figure S2 and Data S1.

(Figure 3A). These results confirm that successful *Caballeronia* colonization induces a behavioral cue in nymphs to switch from searching to feeding, presumably to fuel the growth and development that can only progress with the help of their symbiont.[11] These data highlight how *A. tristis* can shut down searching behavior when it is no longer needed, further reducing the risks associated with environmental transmission.

In addition to symbiont-seeking behavior, we also explored the mechanisms and efficacy of symbiont acquisition. We confirmed that when nymphs successfully find a source of symbionts, they insert their stylets into the solid fecal material and appear to use a lacerate-and-flush feeding technique that liquifies the feces. This behavior, which is typical of seed-feeding Heteroptera,[34,77] presumably facilitates extraction of the embedded beneficial microbes (Video S4) and has not been described in Coreidae before. This feeding technique is an effective symbiont uptake strategy, as 70.6% (60/85) of nymphs in the choice trials that contained symbiont-positive feces (Figure 2D) harbored fluorescent bacteria in their abdomens. Acquisition success was similarly high when the symbiont was not embedded in feces (Figure 3B; Data S1B; GLMM, $P = 0.18$). In these trials, 61.1% (110/180) of nymphs acquired *Caballeronia*, indicating that the feces itself is not necessary for successful colonization.

Although nymphs have clearly evolved the ability to find and consume *Caballeronia*, they still depend on the symbiont being sufficiently available in the environment. The frequency and timing of adult defecation therefore becomes critical for transmission success. In many systems, adults only shed symbionts when they are ready to reproduce.[57,78,79] Females will often deposit symbionts on or near eggs to immediately be ingested by newly hatched offspring.[54,57,58,80] *A. tristis* nymphs, however, do not ingest their symbionts as soon as they hatch. Instead, hatchling nymphs wait 2 days until they molt into their second instar to acquire symbionts.[61] We therefore tested if adult *A. tristis* defecated more when second instar nymphs were present. We found that females shed similar amounts of symbiont-rich feces regardless of the presence or absence of the next generation (t test; n = 34, DF = 29.0, t = 0.40, P = 0.69). We then conducted a complementary experiment to assess how squash bug age influences their ability to transmit symbionts. By tracking transmission dynamics over the course of 12 weeks, which is the typical lifespan of *A. tristis* females,[71] we found that adult females can pass *Caballeronia* to nymphs throughout their entire lives (Figure 3C). Interestingly, data also show that although these females maintain high overall transmission potential as they age, this ability peaks during a period when females are most fecund[65] (Figure 3C; Data S1C; GLMM, $P = 0.0007$). This pattern may correspond with some physiological aspect of host aging, which could impact fecal excretion rate, or alternately, it may reflect changing symbiont titers being excreted. Either way, the consistent output of symbionts in feces ensures that if *A. tristis* adults are around, symbionts should be readily available for nymphs regardless of when and where they hatch.

Last, we explored the fidelity of *A. tristis*' horizonal transmission strategy in more complex, semi-natural communities where nymphs are exposed to different combinations of host species and symbiont strains. Although *Anasa* species in North America have broadly overlapping ranges,[81] a previous study found that two squash bug species, *A. tristis* and *Anasa andresii*, both occupy at a single field site and harbor closely related but distinct strains of *Caballeronia*.[82] Traditionally, this pattern of phylosymbiosis might be interpreted as evidence of host-symbiont co-evolution,[83] where interactions between certain lineages of host and microbe are sustained by reciprocal selection for increasing compatibility. Curiously, however, transplant experiments show no negative consequences for *A. tristis* that are inoculated with *Caballeronia* strains isolated from *A. andresii*.[82] This raises the possibility that mere selection for optimal host-symbiont combinations is not sufficient, on its own, to explain *Anasa-Caballeronia* phylosymbiosis.

We used experimental inoculations to create four types of hosts: (1) *A. tristis* adults harboring the *A. tristis* symbiont strain, (2) *A. tristis* adults harboring the *A. andresii* symbiont strain, (3) *A. andresii* adults harboring the *A. tristis* symbiont strain, or (4)

*A. andresii* adults harboring *A. andresii* symbiont strain (Figure 4). After confirming that both host species can deposit both strains of *Caballeronia* in their feces (Figure S2), we exposed symbiont-free *A. tristis* to one of the four host types. Our design allowed us to decouple the influence of host species and symbiont strain on transmission to *A. tristis.* Nymphs had the highest acquisition success when housed with *A. tristis* adults that shed *A. tristis*-derived symbionts (Figure 4). Among these 10 replicated enclosures, 88.8% (80/90) of nymphs took up the symbiont. However, acquisition success significantly dropped to 71.9% (64/89) when nymphs were instead exposed to *A. tristis* adults that passed an *A. andresii*-derived symbiont (Figure 4; Data S1D; GLMM, $P < 0.0001$). These results were surprising given this strain of *Caballeronia* is no less beneficial than the *tristis*-derived strain for *A. tristis*.[82] Despite the drop in acquisition success, these data confirm that conspecific transmission among *A. tristis* is reliable even when passing different *Caballeronia* strains.

Amazingly, however, transmission success dropped precipitously when nymphs were housed with *A. andresii* symbiont donors. Nymphs were significantly less successful at picking up not only the *A. andresii* symbionts (Figure 4; Data S1D; GLMM, $P < 0.0001$) but their own *A. tristis* symbionts (GLMM, $P < 0.0001$) as well. Of the 190 nymphs involved in the 20 heterospecific transmission cages, only 7 (3.6%) obtained symbionts from *A. andresii* adults. Of these, 5 were from a single cage. This narrow preference for conspecific feces may increase the likelihood that nymphs acquire *Caballeronia* deposited by their own species, even though they may be compatible with many other microbial strains in the environment. These results show that species-specific transmission pathways alone can promote phylosymbiosis, which is consistent with theoretical predictions based on individual mammalian gut taxa,[84] but contrary to conventional assumptions in insect-microbial mutualisms.[85] Future studies should concentrate on the mechanisms hindering heterospecific transmission. Moreover, additional choice and feeding assays should be run to examine *A. andresii* acquisition behaviors to determine how widespread these behaviors are among this group of pests.

Our study provides insight into a longstanding paradox concerning the origin and maintenance of specificity in horizontally transmitted insect-microbial mutualisms. Unlike many studies that have relied on indirect inferences of transmission events, we incorporate direct behavioral observations to reveal how hosts can systematically reduce the risks of obtaining beneficial symbionts in the environment every generation. Overall, our study reveals how specialized behavior not only drives the evolution of a reliable horizontal transmission strategy, but also provides mechanisms that drive patterns of species-specific microbial communities among closely related, sympatric host species.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:



## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.cub.2023.05.062.

## ACKNOWLEDGMENTS

We thank E. Edwards and J. Chavez for all logistical help. We also thank J. de Roode, S. Mendiola, K. Stoy, and the rest of the GerDer group for discussion and other assistance. Funding for this work was supported by a National Institutes of Health K12 Grant (GM00680) as well as a USDA-NIFA Grant (2019-67013-29371) to N.M.G. S.M.V. was also supported by Emory University, an Institutional Research and Academic Career Development Award (IRACDA), and the Fellowships in Research and Science Teaching (FIRST) postdoctoral program.

## AUTHOR CONTRIBUTIONS

S.M.V., J.Z.C., and N.M.G. designed the experiments. J.Z.C., A.A., and Z.K. collected the data. S.M.V. and J.Z.C. analyzed the data. S.M.V. wrote the paper with input from J.Z.C., N.M.V., and N.M.G. All authors agree to be held accountable for the content therein and approve of the final version of the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

Received: December 5, 2022
Revised: April 7, 2023
Accepted: May 25, 2023
Published: June 28, 2023

## REFERENCES

1. Bright, M., and Bulgheresi, S. (2010). A complex journey: transmission of microbial symbionts. Nat. Rev. Microbiol. *8*, 218–230. https://doi.org/10.1038/nrmicro2262.
2. Shapiro, J.W., and Turner, P.E. (2014). The impact of transmission mode on the evolution of benefits provided by microbial symbionts. Ecol. Evol. *4*, 3350–3361. https://doi.org/10.1002/ece3.1166.
3. Wilkinson, D.M., and Sherratt, T.N. (2001). Horizontally acquired mutualisms, an unsolved problem in ecology? Oikos *92*, 377–384. https://doi.org/10.1034/j.1600-0706.2001.920222.x.

4. Genkai-Kato, M., and Yamamura, N. (1999). Evolution of Mutualistic Symbiosis without Vertical Transmission. Theor. Popul. Biol. *55*, 309–323. https://doi.org/10.1006/tpbi.1998.1407.

5. Kikuchi, Y., Meng, X.-Y., and Fukatsu, T. (2005). Gut symbiotic bacteria of the genus Burkholderia in the broad-headed bugs *Riptortus clavatus* and *Leptocorisa chinensis* (Heteroptera: Alydidae). Appl. Environ. Microbiol. *71*, 4035–4043. https://doi.org/10.1128/AEM.71.7.4035-4043.2005.

6. Picazo, D.R., Dagan, T., Ansorge, R., Petersen, J.M., Dubilier, N., and Kupczok, A. (2019). Horizontally transmitted symbiont populations in deep-sea mussels are genetically isolated. Preprint at bioRxiv. https://doi.org/10.1101/536854.

7. Kiers, E.T., Rousseau, R.A., West, S.A., and Denison, R.F. (2003). Host sanctions and the legume–rhizobium mutualism. Nature *425*, 78–81. https://doi.org/10.1038/nature01931.

8. Nyholm, S.V., and McFall-Ngai, M.J. (2004). The winnowing: establishing the squid–vibrio symbiosis. Nat. Rev. Microbiol. *2*, 632–642. https://doi.org/10.1038/nrmicro957.

9. Harii, S., Yasuda, N., Rodriguez-Lanetty, M., Irie, T., and Hidaka, M. (2009). Onset of symbiosis and distribution patterns of symbiotic dinoflagellates in the larvae of scleractinian corals. Mar. Biol. *156*, 1203–1212. https://doi.org/10.1007/s00227-009-1162-9.

10. Peeters, C., Meier-Kolthoff, J.P., Verheyde, B., De Brandt, E., Cooper, V.S., and Vandamme, P. (2016). Phylogenomic Study of *Burkholderia glathei*-like Organisms, Proposal of 13 Novel *Burkholderia* Species and Emended Descriptions of *Burkholderia sordidicola, Burkholderia zhejiangensis*, and *Burkholderia grimmiae*. Front. Microbiol. *7*, 877. https://doi.org/10.3389/fmicb.2016.00877.

11. Acevedo, T.S., Fricker, G.P., Garcia, J.R., Alcaide, T., Berasategui, A., Stoy, K.S., and Gerardo, N.M. (2021). The Importance of Environmentally Acquired Bacterial Symbionts for the Squash Bug (Anasa tristis), a Significant Agricultural Pest. Front. Microbiol. *12*, 719112. https://doi.org/10.3389/fmicb.2021.719112.

12. Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2011). An ancient but promiscuous host–symbiont association between Burkholderia gut symbionts and their heteropteran hosts. ISME J. *5*, 446–460. https://doi.org/10.1038/ismej.2010.150.

13. Kaltenpoth, M., and Flórez, L.V. (2020). Versatile and Dynamic Symbioses Between Insects and Burkholderia Bacteria. Annu. Rev. Entomol. *65*, 145–170. https://doi.org/10.1146/annurev-ento-011019-025025.

14. Dobritsa, A.P., and Samadpour, M. (2016). Transfer of eleven species of the genus Burkholderia to the genus Paraburkholderia and proposal of Caballeronia gen. nov. to accommodate twelve species of the genera Burkholderia and Paraburkholderia. Int. J. Syst. Evol. Microbiol. *66*, 2836–2846. https://doi.org/10.1099/ijsem.0.001065.

15. Dobritsa, A.P., and Samadpour, M. (2019). Reclassification of Burkholderia insecticola as Caballeronia insecticola comb. nov. and reliability of conserved signature indels as molecular synapomorphies. Int. J. Syst. Evol. Microbiol. *69*, 2057–2063. https://doi.org/10.1099/ijsem.0.003431.

16. Haine, E.R. (2008). Symbiont-mediated protection. Proc. Biol. Sci. *275*, 353–361. https://doi.org/10.1098/rspb.2007.1211.

17. Bull, J.J., Molineux, I.J., and Rice, W.R. (1991). Selection of Benevolence in a Host–Parasite System. Evolution *45*, 875–882. https://doi.org/10.1111/j.1558-5646.1991.tb04356.x.

18. Ewald, P.W. (1987). Transmission Modes and Evolution of the Parasitism-Mutualism Continuuma. Ann. N. Y. Acad. Sci. *503*, 295–306. https://doi.org/10.1111/j.1749-6632.1987.tb40616.x.

19. Drew, G.C., Stevens, E.J., and King, K.C. (2021). Microbial evolution and transitions along the parasite–mutualist continuum. Nat. Rev. Microbiol. *19*, 623–638. https://doi.org/10.1038/s41579-021-00550-7.

20. Drown, D.M., Zee, P.C., Brandvain, Y., and Wade, M.J. (2013). Evolution of transmission mode in obligate symbionts. Evol. Ecol. Res. *15*, 43–59.

21. Buchner, P. (1965). Endosymbiosis of Animals with Plant Microorganisms (Interscience Publishers).

22. Koga, R., Meng, X.-Y., Tsuchida, T., and Fukatsu, T. (2012). Cellular mechanism for selective vertical transmission of an obligate insect symbiont at the bacteriocyte–embryo interface. Proc. Natl. Acad. Sci. USA *109*, E1230–E1237. https://doi.org/10.1073/pnas.1119212109.

23. Maire, J., Parisot, N., Galvao Ferrarini, M., Vallier, A., Gillet, B., Hughes, S., Balmand, S., Vincent-Monégat, C., Zaidman-Rémy, A., and Heddi, A. (2020). Spatial and morphological reorganization of endosymbiosis during metamorphosis accommodates adult metabolic requirements in a weevil. Proc. Natl. Acad. Sci. USA *117*, 19347–19358. https://doi.org/10.1073/pnas.2007151117.

24. Rafiqi, A.M., Rajakumar, A., and Abouheif, E. (2020). Origin and elaboration of a major evolutionary transition in individuality. Nature *585*, 239–244. https://doi.org/10.1038/s41586-020-2653-6.

25. Hosokawa, T., Hironaka, M., Mukai, H., Inadomi, K., Suzuki, N., and Fukatsu, T. (2012). Mothers never miss the moment: a fine-tuned mechanism for vertical symbiont transmission in a subsocial insect. Anim. Behav. *83*, 293–300. https://doi.org/10.1016/j.anbehav.2011.11.006.

26. Matsuura, Y., Moriyama, M., Łukasik, P., Vanderpool, D., Tanahashi, M., Meng, X.-Y., McCutcheon, J.P., and Fukatsu, T. (2018). Recurrent symbiont recruitment from fungal parasites in cicadas. Proc. Natl. Acad. Sci. USA *115*, E5970–E5979. https://doi.org/10.1073/pnas.1803245115.

27. Pannebakker, B.A., Loppin, B., Elemans, C.P.H., Humblot, L., and Vavre, F. (2007). Parasitic inhibition of cell death facilitates symbiosis. Proc. Natl. Acad. Sci. USA *104*, 213–215. https://doi.org/10.1073/pnas.0607845104.

28. Salem, H., Onchuru, T.O., Bauer, E., and Kaltenpoth, M. (2015). Symbiont transmission entails the risk of parasite infection. Biol. Lett. *11*, 20150840. https://doi.org/10.1098/rsbl.2015.0840.

29. Pons, I., Renoz, F., Noël, C., and Hance, T. (2019). Circulation of the Cultivable Symbiont Serratia symbiotica in Aphids Is Mediated by Plants. Front. Microbiol. *10*, 764. https://doi.org/10.3389/fmicb.2019.00764.

30. Levinson, H.Z., Levinson, A.R., Müller, B., and Steinbrecht, R.A. (1974). Structure of sensilla, olfactory perception, and behaviour of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. J. Insect Physiol. *20*, 1231–1248. https://doi.org/10.1016/0022-1910(74)90229-7.

31. Brézot, P., Tauban, D., and Renou, M. (1996). Sense organs on the antennal flagellum of the green stink bug, *Nezara viridula* (L.) (Heteroptera : Pentatomidae): Sensillum types and numerical growth during the post-embryonic development. Int. J. Insect Morphol. Embryol. *25*, 427–441. https://doi.org/10.1016/S0020-7322(96)00012-8.

32. Kim, J., Park, K.C., Roh, H.S., Kim, J., Oh, H.W., Kim, J.-A., and Park, C.G. (2016). Morphology and Distribution of Antennal Sensilla of the Bean Bug *Riptortus pedestris* (Hemiptera: Alydidae). Microsc. Res. Tech. *79*, 501–511. https://doi.org/10.1002/jemt.22658.

33. Ventura, M.U., and Panizzi, A.R. (2005). Morphology of olfactory sensilla and its role in host plant recognition by *Neomegalotomus parvus* (Westwood) (Heteroptera: Alydidae). Braz. Arch. Biol. Technol. *48*, 589–597. https://doi.org/10.1590/S1516-89132005000500012.

34. Backus, E.A. (1988). Sensory systems and behaviours which mediate hemipteran plant-feeding: A taxonomic overview. J. Insect Physiol. *34*, 151–165. https://doi.org/10.1016/0022-1910(88)90045-5.

35. Dickens, J.C., Callahan, F.E., Wergin, W.P., and Erbe, E.F. (1995). Olfaction in a hemimetabolous insect: Antennal-specific protein in adult *Lygus lineolaris* (Heteroptera: Miridae). J. Insect Physiol. *41*, 857–867. https://doi.org/10.1016/0022-1910(95)00038-V.

36. Feir, D., and Beck, S.D. (1963). Feeding Behavior of the Large Milkweed Bug, *Oncopeltus fasciatus*. Ann. Entomol. Soc. Am. *56*, 224–229. https://doi.org/10.1093/aesa/56.2.224.

37. Yang, L., Hu, X.P., Allan, S.A., Alborn, H.T., and Bernier, U.R. (2019). Electrophysiological and Behavioral Responses of the Kudzu Bug, *Megacopta cribraria* (Hemiptera: Plataspidae), to Volatile Compounds

from Kudzu and Soybean Plants. J. Agric. Food Chem. *67*, 4177–4183. https://doi.org/10.1021/acs.jafc.8b06765.

38. Roh, G.H., Cha, D.H., and Park, C.G. (2021). Olfactory attraction to aggregation pheromone is mediated by disti-flagellum of antennal segments in *Riptortus pedestris*. J. Asia Pac. Entomol. *24*, 415–420. https://doi.org/10.1016/j.aspen.2021.01.005.
39. Inoue, K., Vidal, D., Saad, E., Martins, C., and Zarbin, P. (2018). Identificaton of the Alarm and Sex Pheromones of the Leaf-Footed Bug, *Leptoglossus zonatus* (Heteroptera: Coreidae). J. Braz. Chem. Soc. *30*, 939–947. https://doi.org/10.21577/0103-5053.20180238.
40. Bohman, B., Weinstein, A.M., Unelius, C.R., and Lorenzo, M.G. (2018). Attraction of *Rhodnius prolixus* males to a synthetic female-pheromone blend. Parasit. Vectors *11*, 418. https://doi.org/10.1186/s13071-018-2997-z.
41. Onnink, C.M., Williams, A.A., and Williams, J.S. (2017). Electrophysiological and Behavioral Evidence for (E)-2-Hexenal as a Female-Attracting Pheromone Produced by Disturbed *Megacopta cribraria* (Hemiptera: Plataspidae). J. Entomol. Sci. *52*, 39–51. https://doi.org/10.18474/JES16-18.1.
42. Zhang, S., Yan, S., Zhang, Z., Cao, S., Li, B., Liu, Y., and Wang, G. (2021). Identification and functional characterization of sex pheromone receptors in mirid bugs (Heteroptera: Miridae). Insect Biochem. Mol. Biol. *136*, 103621. https://doi.org/10.1016/j.ibmb.2021.103621.
43. Hunter, M.S., Umanzor, E.F., Kelly, S.E., Whitaker, S.M., and Ravenscraft, A. (2022). Development of Common Leaf-Footed Bug Pests Depends on the Presence and Identity of Their Environmentally Acquired Symbionts. Appl. Environ. Microbiol. *88*, e01778-21. https://doi.org/10.1128/aem.01778-21.
44. Kim, S., and Lee, D.-H. (2019). Behavioral response of *Riptortus pedestris* (Hemiptera: Alydidae) 2nd instar nymphs to their gut symbiont, *Burkholderia* sp., in laboratory conditions. Entomol. Res. *49*, 265–276. https://doi.org/10.1111/1748-5967.12364.
45. Becher, P.G., Verschut, V., Bibb, M.J., Bush, M.J., Molnár, B.P., Barane, E., Al-Bassam, M.M., Chandra, G., Song, L., Challis, G.L., et al. (2020). Developmentally regulated volatiles geosmin and 2-methylisoborneol attract a soil arthropod to *Streptomyces* bacteria promoting spore dispersal. Nat. Microbiol. *5*, 821–829. https://doi.org/10.1038/s41564-020-0697-x.
46. Huang, H., Ren, L., Li, H., Schmidt, A., Gershenzon, J., Lu, Y., and Cheng, D. (2020). The nesting preference of an invasive ant is associated with the cues produced by actinobacteria in soil. PLoS Pathog. *16*, e1008800. https://doi.org/10.1371/journal.ppat.1008800.
47. Piper, A.M., Farnier, K., Linder, T., Speight, R., and Cunningham, J.P. (2017). Two Gut-Associated Yeasts in a Tephritid Fruit Fly have Contrasting Effects on Adult Attraction and Larval Survival. J. Chem. Ecol. *43*, 891–901. https://doi.org/10.1007/s10886-017-0877-1.
48. Chakraborty, A., Mori, B., Rehermann, G., Hernández Garcia, A., Lemmen-Lechelt, J., Hagman, A., Khalil, S., Håkansson, S., Witzgall, P., and Becher, P.G. (2022). Yeast and fruit fly mutual niche construction and antagonism against mould. Funct. Ecol. *36*, 1639–1654. https://doi.org/10.1111/1365-2435.14054.
49. Shapiro, L., De Moraes, C.M., Stephenson, A.G., Mescher, M.C., and van der Putten, W. (2012). Pathogen effects on vegetative and floral odours mediate vector attraction and host exposure in a complex pathosystem. Ecol. Lett. *15*, 1430–1438. https://doi.org/10.1111/ele.12001.
50. Kenyon, L.J., Meulia, T., and Sabree, Z.L. (2015). Habitat Visualization and Genomic Analysis of "*Candidatus Pantoea carbekii*," the Primary Symbiont of the Brown Marmorated Stink Bug. Genome Biol. Evol. *7*, 620–635. https://doi.org/10.1093/gbe/evv006.
51. Salem, H., Florez, L., Gerardo, N., and Kaltenpoth, M. (2015). An out-of-body experience: the extracellular dimension for the transmission of mutualistic bacteria in insects. Proc. Biol. Sci. *282*, 20142957. https://doi.org/10.1098/rspb.2014.2957.
52. Kaltenpoth, M., Winter, S.A., and Kleinhammer, A. (2009). Localization and transmission route of *Coriobacterium glomerans*, the endosymbiont of pyrrhocorid bugs. FEMS Microbiol. Ecol. *69*, 373–383. https://doi.org/10.1111/j.1574-6941.2009.00722.x.
53. Kikuchi, Y., Hosokawa, T., Nikoh, N., Meng, X.-Y., Kamagata, Y., and Fukatsu, T. (2009). Host-symbiont co-speciation and reductive genome evolution in gut symbiotic bacteria of acanthosomatid stinkbugs. BMC Biol. *7*, 2. https://doi.org/10.1186/1741-7007-7-2.
54. Karamipour, N., Fathipour, Y., and Mehrabadi, M. (2016). Gammaproteobacteria as essential primary symbionts in the striped shield bug, *Graphosoma lineatum* (Hemiptera: Pentatomidae). Sci. Rep. *6*, 33168. https://doi.org/10.1038/srep33168.
55. Hosokawa, T., Ishii, Y., Nikoh, N., Fujie, M., Satoh, N., and Fukatsu, T. (2016). Obligate bacterial mutualists evolving from environmental bacteria in natural insect populations. Nat. Microbiol. *1*, 1–7. https://doi.org/10.1038/nmicrobiol.2015.11.
56. Kaiwa, N., Hosokawa, T., Kikuchi, Y., Nikoh, N., Meng, X.Y., Kimura, N., Ito, M., and Fukatsu, T. (2010). Primary Gut Symbiont and Secondary, *Sodalis*-Allied Symbiont of the Scutellerid Stinkbug *Cantao ocellatus*. Appl. Environ. Microbiol. *76*, 3486–3494. https://doi.org/10.1128/AEM.00421-10.
57. Kaiwa, N., Hosokawa, T., Nikoh, N., Tanahashi, M., Moriyama, M., Meng, X.-Y., Maeda, T., Yamaguchi, K., Shigenobu, S., Ito, M., and Fukatsu, T. (2014). Symbiont-Supplemented Maternal Investment Underpinning Host's Ecological Adaptation. Curr. Biol. *24*, 2465–2470. https://doi.org/10.1016/j.cub.2014.08.065.
58. Prado, S.S., Rubinoff, D., and Almeida, R.P.P. (2006). Vertical Transmission of a Pentatomid Caeca-Associated Symbiont. Ann. Entomol. Soc. Am. *99*, 577–585. https://doi.org/10.1603/0013-8746(2006)99[577:VTOAPC]2.0.CO;2.
59. Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2007). Insect-Microbe Mutualism without Vertical Transmission: a Stinkbug Acquires a Beneficial Gut Symbiont from the Environment Every Generation. Appl. Environ. Microbiol. *73*, 4308–4316. https://doi.org/10.1128/AEM.00067-07.
60. Ohbayashi, T., Takeshita, K., Kitagawa, W., Nikoh, N., Koga, R., Meng, X.-Y., Tago, K., Hori, T., Hayatsu, M., Asano, K., et al. (2015). Insect's intestinal organ for symbiont sorting. Proc. Natl. Acad. Sci. USA *112*, E5179–E5188. https://doi.org/10.1073/pnas.1511454112.
61. Kikuchi, Y., Hosokawa, T., and Fukatsu, T. (2011). Specific Developmental Window for Establishment of an Insect-Microbe Gut Symbiosis. Appl. Environ. Microbiol. *77*, 4075–4081. https://doi.org/10.1128/AEM.00358-11.
62. Itoh, H., Jang, S., Takeshita, K., Ohbayashi, T., Ohnishi, N., Meng, X.-Y., Mitani, Y., and Kikuchi, Y. (2019). Host–symbiont specificity determined by microbe–microbe competition in an insect gut. Proc. Natl. Acad. Sci. USA *116*, 22673–22682. https://doi.org/10.1073/pnas.1912397116.
63. Kikuchi, Y., Ohbayashi, T., Jang, S., and Mergaert, P. (2020). *Burkholderia insecticola* triggers midgut closure in the bean bug *Riptortus pedestris* to prevent secondary bacterial infections of midgut crypts. ISME J. *14*, 1627–1638. https://doi.org/10.1038/s41396-020-0633-3.
64. Caspi-Fluger, A., Inbar, M., Mozes-Daube, N., Katzir, N., Portnoy, V., Belausov, E., Hunter, M.S., and Zchori-Fein, E. (2012). Horizontal transmission of the insect symbiont *Rickettsia* is plant-mediated. Proc. Biol. Sci. *279*, 1791–1796. https://doi.org/10.1098/rspb.2011.2095.
65. Gonella, E., Crotti, E., Rizzi, A., Mandrioli, M., Favia, G., Daffonchio, D., and Alma, A. (2012). Horizontal transmission of the symbiotic bacterium *Asaia* sp. in the leafhopper *Scaphoideus titanus* Ball (Hemiptera: Cicadellidae). BMC Microbiol. *12*, S4. https://doi.org/10.1186/1471-2180-12-S1-S4.
66. Nishino, T., Hosokawa, T., Meng, X.-Y., Koga, R., Moriyama, M., and Fukatsu, T. (2021). Environmental Acquisition of Gut Symbiotic Bacteria in the Saw-Toothed Stinkbug, *Megymenum gracilicorne* (Hemiptera: Pentatomoidea: Dinidoridae). jzoo *38*, 213–222. https://doi.org/10.2108/zs200163.
67. Simms, E.L., Taylor, D.L., Povich, J., Shefferson, R.P., Sachs, J.L., Urbina, M., and Tausczik, Y. (2006). An empirical test of partner choice

mechanisms in a wild legume-rhizobium interaction. Proc. Biol. Sci. *273*, 77–81. https://doi.org/10.1098/rspb.2005.3292.

68. Wang, Q., Liu, J., and Zhu, H. (2018). Genetic and Molecular Mechanisms Underlying Symbiotic Specificity in Legume-Rhizobium Interactions. Front. Plant Sci. *9*, 313.
69. Roy, P., Achom, M., Wilkinson, H., Lagunas, B., and Gifford, M.L. (2020). Symbiotic Outcome Modified by the Diversification from 7 to over 700 Nodule-Specific Cysteine-Rich Peptides. Genes *11*, 348. https://doi.org/10.3390/genes11040348.
70. Yang, S., Wang, Q., Fedorova, E., Liu, J., Qin, Q., Zheng, Q., Price, P.A., Pan, H., Wang, D., Griffitts, J.S., et al. (2017). Microsymbiont discrimination mediated by a host-secreted peptide in *Medicago truncatula*. Proc. Natl. Acad. Sci. USA *114*, 6848–6853. https://doi.org/10.1073/pnas.1700460114.
71. Wang, Q., Yang, S., Liu, J., Terecskei, K., Ábrahám, E., Gombár, A., Domonkos, Á., Szűcs, A., Körmöczi, P., Wang, T., et al. (2017). Host-secreted antimicrobial peptide enforces symbiotic selectivity in *Medicago truncatula*. Proc. Natl. Acad. Sci. USA *114*, 6854–6859. https://doi.org/10.1073/pnas.1700715114.
72. Nishiguchi, M.K., Ruby, E.G., and McFall-Ngai, M.J. (1998). Competitive Dominance among Strains of Luminous Bacteria Provides an Unusual Form of Evidence for Parallel Evolution in Sepiolid Squid-*Vibrio* Symbioses. Appl. Environ. Microbiol. *64*, 3209–3213.
73. Nishiguchi, M.K. (2002). Host-symbiont recognition in the environmentally transmitted sepiolid squid-*Vibrio* mutualism. Microb. Ecol. *44*, 10–18. https://doi.org/10.1007/BF03036870.
74. Chavez-Dozal, A.A., Gorman, C., Lostroh, C.P., and Nishiguchi, M.K. (2014). Gene-Swapping Mediates Host Specificity among Symbiotic Bacteria in a Beneficial Symbiosis. PLoS One *9*, e101691. https://doi.org/10.1371/journal.pone.0101691.
75. Russell, J.A., and Moran, N.A. (2005). Horizontal Transfer of Bacterial Symbionts: Heritability and Fitness Effects in a Novel Aphid Host. Appl. Environ. Microbiol. *71*, 7987–7994. https://doi.org/10.1128/AEM.71.12.7987-7994.2005.
76. Villa, S.M., Han, D.D., Jordan, M.E., and Gerardo, N.M. (2021). The resilience of reproductive interference. Evol. Ecol. *35*, 537–553. https://doi.org/10.1007/s10682-021-10120-1.
77. Miles, P.W. (1972). The Saliva of Hemiptera. In Advances in Insect Physiology, J.E. Treherne, M.J. Berridge, and V.B. Wigglesworth, eds. (Academic Press), pp. 183–255. https://doi.org/10.1016/S0065-2806(08)60277-5.
78. Koga, R., Tanahashi, M., Nikoh, N., Hosokawa, T., Meng, X.-Y., Moriyama, M., and Fukatsu, T. (2021). Host's guardian protein counters degenerative symbiont evolution. Proc. Natl. Acad. Sci. USA *118*. e2103957118. https://doi.org/10.1073/pnas.2103957118.
79. Vigneron, A., Masson, F., Vallier, A., Balmand, S., Rey, M., Vincent-Monégat, C., Aksoy, E., Aubailly-Giraud, E., Zaidman-Rémy, A., and Heddi, A. (2014). Insects Recycle Endosymbionts when the Benefit Is Over. Curr. Biol. *24*, 2267–2273. https://doi.org/10.1016/j.cub.2014.07.065.
80. Flórez, L.V., Scherlach, K., Gaube, P., Ross, C., Sitte, E., Hermes, C., Rodrigues, A., Hertweck, C., and Kaltenpoth, M. (2017). Antibiotic-producing symbionts dynamically transition between plant pathogenicity and insect-defensive mutualism. Nat. Commun. *8*, 15172. https://doi.org/10.1038/ncomms15172.
81. Brailovsky, H. (1985). Revisión del género *Anasa* Amyot-Serville (Hemiptera, Heteroptera, Coreidae, Coreini) (Universidad Nacional Autónoma de México).
82. Stoy, K., Chavez, J., Casas, V.D.L., Talla, V., Berasategui, A., Morran, L., and Gerardo, N. (2021). Evaluating the role of coevolution in a horizontally transmitted mutualism. Preprint at bioRxiv. https://doi.org/10.1101/2021.12.04.471243.
83. Brooks, A.W., Kohl, K.D., Brucker, R.M., van Opstal, E.J., and Bordenstein, S.R. (2016). Phylosymbiosis: Relationships and Functional Effects of Microbial Communities across Host Evolutionary History. PLoS Biol. *14*, e2000225. https://doi.org/10.1371/journal.pbio.2000225.
84. Groussin, M., Mazel, F., and Alm, E.J. (2020). Co-evolution and Co-speciation of Host-Gut Bacteria Systems. Cell Host Microbe *28*, 12–22. https://doi.org/10.1016/j.chom.2020.06.013.
85. Kohl, K.D. (2020). Ecological and evolutionary mechanisms underlying patterns of phylosymbiosis in host-associated microbial communities. Philos. Trans. R. Soc. Lond. B Biol. Sci. *375*, 20190251. https://doi.org/10.1098/rstb.2019.0251.
86. Miller, V.L., and Mekalanos, J.J. (1988). A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in Vibrio cholerae requires toxR. J. Bacteriol. *170*, 2575–2583. https://doi.org/10.1128/jb.170.6.2575-2583.1988.
87. Wiles, T.J., Wall, E.S., Schlomann, B.H., Hay, E.A., Parthasarathy, R., and Guillemin, K. (2018). Modernized Tools for Streamlined Genetic Manipulation and Comparative Study of Wild and Diverse Proteobacterial Lineages. mBio *9*, 01877-18. https://doi.org/10.1128/mBio.01877-18.
88. Choi, K.-H., Gaynor, J.B., White, K.G., Lopez, C., Bosio, C.M., Karkhoff-Schweizer, R.R., and Schweizer, H.P. (2005). A Tn7-based broad-range bacterial cloning and expression system. Nat. Methods *2*, 443–448. https://doi.org/10.1038/nmeth765.
89. Friard, O., and Gamba, M. (2016). BORIS: a free, versatile open-source event-logging software for video/audio coding and live observations. Methods Ecol. Evol. *7*, 1325–1330. https://doi.org/10.1111/2041-210X.12584.
90. Pédelacq, J.D., Cabantous, S., Tran, T., Terwilliger, T.C., and Waldo, G.S. (2006). Engineering and characterization of a superfolder green fluorescent protein. Nat. Biotechnol. *24*, 79–88. https://doi.org/10.1038/nbt1172.
91. Shaner, N.C., Campbell, R.E., Steinbach, P.A., Giepmans, B.N.G., Palmer, A.E., and Tsien, R.Y. (2004). Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. Nat. Biotechnol. *22*, 1567–1572. https://doi.org/10.1038/nbt1037.
92. de Boer, H.A., Comstock, L.J., and Vasser, M. (1983). The *tac* promoter: a functional hybrid derived from the *trp* and *lac* promoters. Proc. Natl. Acad. Sci. USA *80*, 21–25.
93. Koch, B., Jensen, L.E., and Nybroe, O. (2001). A panel of Tn7-based vectors for insertion of the gfp marker gene or for delivery of cloned DNA into Gram-negative bacteria at a neutral chromosomal site. J. Microbiol. Methods *45*, 187–195. https://doi.org/10.1016/S0167-7012(01)00246-9.
94. Kikuchi, Y., and Fukatsu, T. (2014). Live imaging of symbiosis: spatiotemporal infection dynamics of a GFP-labelled *Burkholderia* symbiont in the bean bug *Riptortus pedestris*. Mol. Ecol. *23*, 1445–1456. https://doi.org/10.1111/mec.12479.
95. Bao, Y., Lies, D.P., Fu, H., and Roberts, G.P. (1991). An improved Tn7-based system for the single-copy insertion of cloned genes into chromosomes of gram-negative bacteria. Gene *109*, 167–168. https://doi.org/10.1016/0378-1119(91)90604-A.
96. (2010). M9 minimal medium (standard). Cold Spring Harb. Protoc. *2010*. pdb.rec12295. https://doi.org/10.1101/pdb.rec12295.
97. Bates, D.W., Mächler, M., Bolker, B., and Walker, S. (2015). Fitting Linear Mixed-Effects Models Using lme4. BMJ Qual. Saf. *24*, 1–3. https://doi.org/10.18637/jss.v067.i01.
98. R Core Team (2022). R: A Language and Environment for Statistical Computing (R Foundation for Statistical Computing).
99. Kuznetsova, A., Brockhoff, P.B., and Christensen, R.H.B. (2017). lmerTest Package: Tests in Linear Mixed Effects Models. J. Stat. Softw. *82*, 1–26. https://doi.org/10.18637/jss.v082.i13.
100. Delignette-Muller, M.L., and Dutang, C. (2015). fitdistrplus: An R Package for Fitting Distributions. J. Stat. Softw. *64*, 1–34. https://doi.org/10.18637/jss.v064.i04.

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062



## STAR★METHODS

### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Bacterial and virus strains | | |
| *Caballeronia* sp. strain GA-OX1 | Acevedo et al.[11] | N/A |
| *Caballeronia* sp. strain AAF182 | Stoy et al.[82] | N/A |
| *Escherichia coli* SM10(λpir) | Miller and Mekalanos[86] via Wiles et al.[87] | LMBP 3889 |
| Experimental models: Organisms/strains | | |
| *Anasa tristis* | Acevedo et al.[11] | N/A |
| *Anasa andresii* | Villa et al.[76] | N/A |
| Oligonucleotides | | |
| Primer: glmS-1434F AGGCGCGTTGAAGCTCAAGG | This study | N/A |
| Primer: aacC-83F GTATGCGCTCACGCAACTGG | This study | N/A |
| Primer: lacI-3644F TCGCAGAGTATGCCGGTGTC | This study | N/A |
| Recombinant DNA | | |
| pTn7xKS-sfGFP | Wiles et al.[87] | Addgene plasmid 117394 |
| pTn7xKS-dTomato | Wiles et al.[87] | Addgene plasmid 117395 |
| pTNS2 | Choi et al.[88] via Wiles et al.[87] | Addgene plasmid 64968 |
| Software and algorithms | | |
| Olympus cellSens Standard software ver. 1.13 | Olympus Corporation | https://www.olympus-lifescience.com/en/software/cellsens/ |
| LAS X ver. 3.4.2.18368 | Leica Microsystems GmbH | https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/ |
| BORIS ver. 7.12.2 | Friard & Gamba (2016)[89] | https://www.boris.unito.it/ |

### RESOURCE AVAILABILITY

#### Lead contact

All inquiries and requests for materials should be directed to, and will be fulfilled by, the lead contact, Scott Villa at scott.villa@gmail.com.

#### Materials availability

This study did not generate any new, unique reagents.

#### Data and code availability

- All data are available in the figures, tables, and data files associated with this manuscript.
- This study did not result in any unique code.
- Any additional information required to re-analyze the data reported in this paper is available from the lead contact upon request.

### EXPERIMENTAL MODEL AND SUBJECT DETAILS

Squash bugs (*Anasa tristis* and *A. andresii*) are reared as described in Acevedo et al. (2021).[11] The squash bug lab colonies are descended from bugs collected in Gainesville, Florida, to which wild bugs from the original source populations are added every year to maintain genetic diversity. Bugs are maintained at 27°C–30°C with a day/night cycle of 16 h light/8 h darkness. Five to ten adults are reared in 12 × 12 × 12 inch cubic mesh cages, each containing a potted summer squash (*Cucurbita pepo* "Goldstar") plant, which is changed out as needed. Egg clutches are collected every three to four days, surface sterilized with successive washes of 70%

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062





denatured ethanol, 20% bleach, and DI water, and air dried in plastic rearing containers in a laminar flow hood. First instar nymphs, which do not need to feed but require moisture, are maintained on pieces of organic zucchini squash wrapped in Parafilm, on which they molt to the second instar in two days. Second instars to be used in experiments are drawn from this pool and are maintained on zucchini for no more than one week.

*Caballeronia* sp. strain GA-OX1 was previously isolated from homogenized M4 crypts dissected from an adult squash bug (*Anasa tristis*) collected in Oxford, Georgia.[11] *Caballeronia* sp. strain AAF182 was previously isolated in the same manner from a female squash bug in the community organic garden at the University of Florida.[82] For experiments, strains are streaked out onto nutrient agar plates from 25% glycerol stocks stored at −80°C, and incubated at 30°C for two days. To prepare liquid cultures, 3 mL salt-free LB (Luria-Bertani) broth cultures are inoculated with a single colony from each plate streak and grown to log phase at 30°C shaking at 225 rpm overnight.

## METHOD DETAILS

### Fluorescent-labeling of *Caballeronia* strains

The mini-Tn7 system[88] enables site- and orientation-specific insertion of recombinant DNA in an intergenic region 20–25 base pairs downstream of the glmS gene in eubacteria. We used modified mini-Tn7 vectors[87] to label *Caballeronia* strains with superfolder GFP[90] (henceforth GFP) and dTomato[91] (henceforth RFP). Crucially, the *tac* promoter[92] and an efficient ribosomal binding site (iGem part BBa_K2306014) in these mini-Tn7 constructs drives stronger fluorophore expression than constructs previously utilized in *Caballeronia* (i.e. Koch et al. 2001[93]), enabling rapid screening of live or whole bugs up to adulthood for symbiotic infection without dissection. Furthermore, the gentamicin resistance marker, combined with inducible toxic peptides on the plasmid backbone,[87] enables efficient selection for transconjugants without relying on lab-evolved antibiotic resistant mutants as target strains.[94] The modern standard helper plasmid that provides the genomic integration function *in trans* contains only the TnsABC+D pathway for *att*Tn7-specific integration, unlike obsolete plasmids that encode the additional TnsABC+E non-specific integration function.[95]

Triparental conjugations were conducted as follows. *Escherichia coli* SM10(λpir) carrying the donor plasmids pTn7xKS-sfGFP and pTn7xKS-dTomato, as well as the helper plasmid pTNS2, were a generous gift from Dr. Travis Wiles.[87] *Caballeronia* liquid cultures were grown as described above. Donor and helper SM10(λpir) were grown overnight in LB broth with 150 μg/mL ampicillin for selection at 37°C shaking at 225 rpm. Donor and helper cultures were washed at least twice to remove residual ampicillin by repeatedly pelleting at 10,000 xg for 2 min at 4°C, decanting the supernatant, and resuspending in the same volume of LB. These were combined with washed *Caballeronia* on LB agar containing 1% NaCl to mate for 24–50 h at 30°C. Matings were harvested into salt-free LB broth, subjected to selection with lytic T7 phage for 4 h to eradicate *E. coli*, then plated on low-salt LB or M9 + 0.4% glucose[96] agar plates, both containing 1mM IPTG and 5–10 μg/mL gentamicin for selection. Fluorescent colonies were streaked on nutrient agar to confirm stability of fluorophore expression, and correct insertion of the mini-Tn7 construct was confirmed by PCR amplifying the fragment bridging the *glmS* gene and the gentamicin resistance marker aacC, using primers glmS-1434F and aacC-83F. The absence of the plasmid backbone in these strains was confirmed by PCR amplifying the fragment bridging aacC and the lacI gene, using primers aacC-83F and lacI-3644F.

### Preparation of donor insects

Eggs were collected from lab colonies of *A. tristis* and *A. andresii*, surface sterilized, and air dried as described above. To maintain the sterility of the bugs prior to infection, hatchlings were maintained on droplets of DI water until they molted to the second instar, which takes place two days after hatching. Second instar nymphs were subsequently maintained on ~100 μL of 2% glucose in 10% PBS solution, which was provided as droplets every 24 h, until symbiont inoculation. Nymphs were not maintained on this sterile diet for more than a week.

To prepare symbiont inocula, overnight liquid cultures were started from single colony picks as described above. 200 μL of log-phase culture was then washed twice in phosphate-buffered saline (PBS), by pelleting for 2 min at 10,000 ×g and decanting the supernatant. Inocula were prepared by diluting these washed cells 100-fold in 400 μL of feeding solution (2% glucose in 10% PBS), and spotting droplets of these inocula in plastic rearing boxes that housed second instar nymphs that had been starved for ~24 h. Nymphs typically imbibed these droplets readily, but were exposed to inocula for 24 h before being transferred to parafilmed cubes of surface-sterilized zucchini. Bugs that did not feed typically starved to death, but colonization was verified by anesthetizing second instars after 2–3 days with carbon dioxide and checking nymphs individually for fluorescence. Successfully infected bugs were reared, segregated by host species and symbiont strain, to adulthood on summer squash plants in mesh cages, as described above.

### Microscopy

Images of bacterial colonies and fecal spots, as well as videos of feeding, were taken on an Olympus SZX16 stereomicroscope with an Olympus XM10 monochrome camera and Olympus cellSens Standard software ver. 1.13. For rapid phenotyping of nymphs for GFP and RFP symbiont colonization, nymphs were immobilized with carbon dioxide or killed with 70% ethanol, then individually examined at the SZX16 stereomicroscope for GFP and RFP signal with the camera set to autoexposure.

Brightfield images of second instar *A. tristis* antennae were taken using whole nymphs were killed in 70% ethanol and mounted on depression slides. Images were taken on a Leica DMi8 inverted widefield light microscope with a Leica DFC9000 GT fluorescence camera and Leica Application Suite X ver. 3.4.2.18368 software.

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062



### Choice assays

Our initial observations showed that second instar nymphs kept in plastic rearing boxes exhibited a strong attraction to feces in the bedding used to line adult rearing cages. To characterize the magnitude of this attraction and the cues that elicited this response, we set up choice assays in bleach- and ethanol-sterilized 11.2 cm × 11.2 cm × 3.9 cm plastic rearing boxes with a piece of parafilm covered organic zucchini squash in the middle of the arena and two choices (fecal spot vs. PBS, autoclaved fecal spot vs. PBS, cultured symbiont vs. PBS, and PBS vs. PBS) in opposing corners of the boxes. The symbiont strain used as the autoclaved or untreated fecal spot and as the cultured bacterial treatment in all these choice assays was always GA-OX1 sfGFP-V10, to control for strain-specific effects. For cultured symbiont vs. PBS, a black mark was placed on the side of the box with the cultured symbiont to distinguish between the two treatments. The control filter paper was cut to a size similar to the paper towels containing fecal spots. Five second instar nymphs were placed onto the squash piece, and boxes were recorded with two boxes under each camera. Orientation and order of each box was decided using a random number generator. To prevent interactions between nymphs in adjacent boxes, white tape was applied to the adjoining side of the boxes. All trials were recorded using overhead HD cameras for 24 or 48 h in a humidity and light-controlled room. Using second instar nymphs with ablated antennae to specifically test the role of olfaction, we conducted additional cultured symbiont vs. PBS, fecal spot vs. PBS and autoclaved fecal spots vs. PBS. Assays were recorded continuously using high-definition Owl AHD10–841-B cameras, equipped with infrared bulbs to film in complete darkness. Videos were exported as separate ~17 min files that were stitched together using MacX Video Converter Pro. After assays were completed, nymphs were allowed to develop for several more days and checked for GFP colonization by visual inspection of whole and dissected nymphs.

### Fecal spot collection and sterilization

Paper towels were used to line the bottom of cages where we maintained donor *Anasa tristis* adults infected with GA-OX1 sfGFP-V10. We checked these paper towels every one to two days for dark, semisolid, fecal spots, which are distinct from the clear, watery feces excreted more frequently by bugs during feeding. As soon as fecal spots were identified, they were cut out from the surrounding paper towel and immediately used in a choice assay or sterilized. Collected paper towel cutouts with fecal spots were sterilized by autoclaving in a glass beaker on a gravity cycle (sterilization cycle of 121°C for 30 min with a dry cycle of 10 min.).

### Cultured bacteria

2.5 mL of overnight culture were washed twice in phosphate-buffered saline (PBS), by pelleting for 2 min at 10,000 xg and decanting the supernatant. This pellet was then resuspended in 2.5 mL of PBS, bringing the washed cells back to their original concentration. For assays comparing PBS to cultured bacteria, 150 μL of washed cells were spotted onto a quartered qualitative filter paper disc (diameter 55 mm) in one corner of each arena, and the same amount of PBS was spotted onto a quarter filter paper disc in the opposite corner. Serial dilution of the inocula *post hoc* confirmed that cells had high viability at the time they were used in the assays.

### Antennal ablation

We chose to ablate only the fourth antennal segment, rather than the whole antenna (Figure S1), because 1) nymphs with completely ablated antennae exhibited extremely poor survival, impaired mobility, and reduced activity; and 2) microscopy showed that the fourth antennal segment is covered in an unusually high density of sensilla relative to the rest of the antennal segments, in line with observations from related insects.[32] Nymphs were briefly anesthetized with carbon dioxide then placed into a glass dish with sterile DI water to immobilize them. The fourth antennal segment was removed using pairs of fine metal forceps. To minimize damage to the rest of the antennae, one set of forceps gently grasped the fourth antennal segment, or the distal part on the third antennal segment, while the second set of forceps was run across the second forceps to sever the fourth segment cleanly at the intersegmental membrane. Control bugs were treated in the same way as ablated bugs, but antennal segments were gently manipulated without being severed. Ablated and control bugs were returned to rearing boxes lined with paper towels to dry off and allowed to recover for two days before being used in choice assays.

### Video analysis

We measured searching behavior in the video-recorded choice assays using Behavioral Observation Research Interactive Software (BORIS) ver. 7.12.2, an open-source event logging software for live observations of animal behavior.[89] Because each arena contained 5 nymphs, the ethogram was set up with 6 point events, ranging from 0 bugs to 5 bugs. Additionally, each event had 2 modifiers corresponding to the 2 attractants in each arena (feces vs. PBS, cultured bacteria vs. PBS, PBS vs. PBS). Video frames were then evaluated at 10-minute intervals for the entire 24-hour period and the number of bugs sitting on filter paper laden with each attractant was recorded. To quantify searching behavior over time, the number of bugs wandering off the squash was counted at 10-minute intervals for the entire 48-h time period, with 0 indicating that all bugs were on the squash in each frame.

### Fecal spot elicitation

We wanted to test if adult fecal production is socially influenced by exposure to nymphs. To test this, same-sex pairs of adult donors infected with the same symbiont were placed in plastic rearing boxes, to which either 5 s instar nymphs were added or no nymphs were added. Each box was lined with paper towels, which were monitored daily for 11 consecutive days for the appearance of dark fecal spots.

Please cite this article in press as: Villa et al., Specialized acquisition behaviors maintain reliable environmental transmission in an insect-microbial mutualism, Current Biology (2023), https://doi.org/10.1016/j.cub.2023.05.062





### Age-dependent transmission

We sought to determine whether ability to transmit symbionts changed during the lifespan of adult squash bugs. Four replicate cages were set up each with four *Anasa tristis* donors infected with RFP symbionts (due to the higher intensity RFP signal) as described above. To prevent mating and nymphal recruitment within these cages, and to control for sex, donors were sexed as fifth instar nymphs, just prior to adulthood, and only female nymphs were kept to be used in experiments as donors. Groups of donors were passaged to new autoclaved cages with new plants and clean water on a weekly basis to prevent symbiont transmission via accumulated biological material over the course of the experiment. The experiment was terminated after 12 weeks, the typical lifetime of unmated *A. tristis* females.[76]

Cohorts of nymphs were assessed for colonization rate within the four replicate cages on a weekly basis. Ten aposymbiotic L1 or L2 nymphs were introduced and allowed to develop. During the weekly passaging of adult donors, live nymphs were harvested, euthanized with 70% alcohol, and checked for RFP under a NightSEA RFP filter attached to a dissecting microscope.

### Interspecific transmission mesocosm assays

Cages surrounding a single potted summer squash plant were set up containing either four adult donor *A. tristis* or four adult donor *A. andresii*, reared as described above and colonized with either GFP- or RFP-labeled GA-OX1 or AAF182. After 5 days, 10 first to second instar nymphs were introduced into the cages; adults continued to be maintained on the plant. (First instars are a two-day long non-foraging stage, but were included for use in these assays because eggs are only synchronized every three to four days.) After 10 days, surviving nymphs were collected from the cages, killed in 70% EtOH, and individually assessed for presence/absence of GFP and RFP. Experiments were started in blocks to ensure that we could compare across treatments.

## QUANTIFICATION AND STATISTICAL ANALYSIS

Preferences in choice assays (Figure 2) were assessed using a series of paired t tests, where the total number of visits to each choice was quantified for every trial.

We analyzed symbiont transmission dynamics using a series of generalized linear mixed models (GLMMs) (Figure 3; Datas S1A–S1C). First, we used a GLMM with Poisson distribution to model searching behavior as a function of treatment (with or without symbionts), time in the trial, and their interaction (Figure 3A; Data S1A). We included trial as a random effect to account for the repeated measures of each trial over the course of the experiment. The intercept of the model was set to the choice trials at the end of the experiment. Next, we model transmission success as a function of symbiont availability (Figure 3B; Data S1B). Because transmission success was scored as ''yes'' or ''no'' for each nymph, we used a GLMM assuming a binomial distribution and logit link function. Trial length was included as a random effect to account for any influence of time spent in the trial on likelihood of picking up a symbiont. Finally, we used a GLMM with binomial distribution and logit link function to model transmission success as a function of female age (Figure 3C; Data S1C). We evaluated both a linear and quadratic relationship between transmission and female age. We included group as a random effect to account for repeated measures of transmission success in each cage over time.

We analyzed transmission specificity using a generalized linear model (GLM) with binomial distribution (Figure 4; Data S1D). We assessed the likelihood that a nymph would pick up symbionts as a function of the donor type. Doner type consisted of a combination of host species and symbiont strain. Post hoc tests were run to account for multiple comparisons.

All models were fit in R using the 'lme4' library package[97,98] (Bates et al. 2015; R Core Team 2016). Degrees of freedom and resulting p values were estimated with the Satterwhite approximation using the lmerTest library[99] (Kuzentsova et al. 2016). When post hoc comparisons between treatments were performed, we used a Tukey's honesty significant difference test (HSD) using the R library package 'emmeans'. The distribution that best fit the data for each model was determined using the 'fitdisplus' package v.1.1e12[100] (Delignette-Muller and Dutang, 2015).