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Transfer of aphid secondary symbionts within sympatric insect communities

and the effect of ingesting symbionts on ladybird beetles

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Abstract

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Aphids cannot survive on their limited diet of plant phloem without the help of their primary symbiotic bacterium, *Buchnera*. In addition to this obligate relationship, a number of facultative symbionts can inhabit this sap-sucking insect. While efficient transmission from mother to daughter is readily seen in the lab for both types, secondary symbionts are thought to undergo horizontal transfer across species in the field. Genetic comparisons suggest these jumps may even occur between very distantly related species, such as whiteflies or ladybirds. Such horizontal transfer could play a critical role in both understanding the colonization of new insect lineages with novel bacteria and in the general understanding of bacterial migration. I explored the transfer among insects in the aphid community, screening field samples of sympatric ants, ladybirds and aphids for three common aphid secondary symbionts and conducting feeding experiments with live ladybirds. I found that ladybird larvae fed aphids with symbionts were half as likely to die as larvae fed aphids without symbionts, suggesting there is an advantage to consuming symbionts. There was decrease in the persistence over time of the symbiont *Serratia symbiotica* in adult ladybird beetles fed aphids with the bacteria, until no bacterial DNA was seen in the ladybirds after one week. No ants or ladybirds were found in the field with any of the three main aphid symbionts, suggesting that horizontal transfer is a rare event. Still, ingestion may need to be considered as a route of symbiont acquisition.

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Alexandra Kamins 2009

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INTRODUCTION

Animals and other eukaryotic organisms provide an excellent habitat for bacteria. However many of even the most successfully reproducing bacteria would quickly vanish without a method to infect new hosts upon the host's death. In order to just survive, many bacterial species must colonize at least one host before the originating infection ends or the initial habitat is lost when the host dies (Wolfe *et al*. 2007). Bacterial species dependent upon other organisms for reproduction and survival have accordingly developed efficient and varied transmission methods, including sexual transmission, infection of developing young and eggs or direct ingestion from the environment (Darby & Douglas 2003).

Patterns of transmission therefore play a vital role in understanding the bacterial ecology, but are to study. The majority of bacterial species are benign, and many species actually offer a benefit to the species they infect (Moran 2006). Known as symbionts, these bacteria infect host species from termites to humans. A growing body of literature examine the common symbiotic bacterial species associated with the pea aphid *Acyrthosiphon pisum*, a sap-sucking agricultural pest; these insects and their surrounding communities provide an excellent microcosm in which to examine and manipulate bacterial transfers.

Aphids and company

Aphids, once a European pest, now exist nearly worldwide in largely temperate regions. These tiny insects parasitize plants, with species usually specific to host plant groups such as pea plants or sycamore and oak trees (Dixon 1997). The aphid diet of phloem is nutritionally very poor, consisting mainly of sugars and unbalanced proportions of amino acids (Liadouze *et al*. 1994). Some species rotate plant host species or migrate to new feeding places; aphids are capable of migrating up to 1300 km on the wind (Dixon 1997). The typical aphid life cycle, as depicted in Figure 1, consists of rapid parthenogenesis from asexual females in the spring until autumn, when the shortened photoperiod triggers the production of sexual males and females, which mate and produce over-wintering eggs (Moran & Dunbar 2006). These eggs then hatch into new asexual females in the spring and begin the cycle anew. In addition to sexual morphs, aphids can also produce winged and unwinged

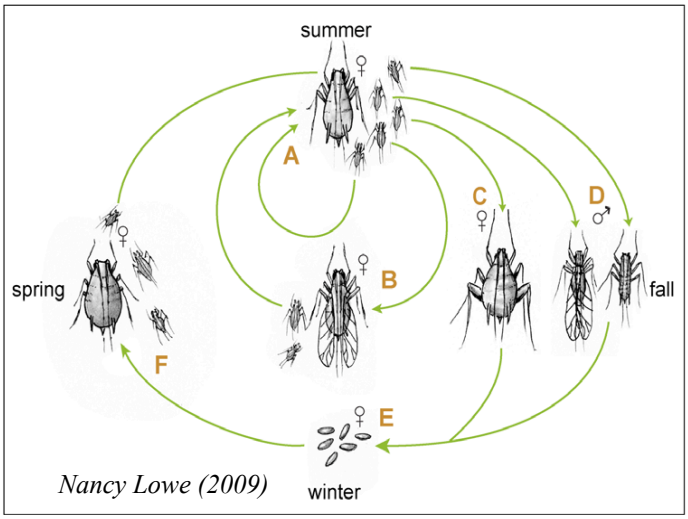

Figure 1: The seasonal cycle of the Pea aphid.

morphs as a consequence of overcrowding or poor conditions, or in response to predation (Minoretti & Weisser 2000).

Nearly all of the 4000 known species of aphids host an obligate symbiont, *Buchnera aphidicola* (Buchner 1965). This bacterium has lost the ability to live independently, while the aphid host has come to rely heavily on its presence and the essential amino acids it synthesizes (Moran 2006). Without this essential bacterial companion, pea aphids suffer from retarded growth, sterility and even death (Koga *et al.* 2003)**.** *Buchnera* is transmitted quite reliably from mother to daughters, and its present distribution and genetic diversity support strict vertical transmission since a common ancestor 84-164 million years ago (Russell *et al*. 2003). Specialized host cells, called mycetocytes or bacteriocytes, house *Buchnera* inside the aphid.

In addition to this primary symbiont, many aphids host secondary symbionts not necessary for, but that often contribute to, survival or reproduction (Russell *et al*. 2003; Moran 2006). Pea aphids worldwide have been found to host at least five vertically transmitted secondary symbionts (Oliver *et al*. 2005). These symbionts confer a variety of benefits to their hosts, including increased heat tolerance, supplementation of essential amino acids, increased resistance against parasitoid wasps and improved ingestion of sucrose (Wilinson & Ishikawa 1998; Oliver *et al*. 2003; Russell & Moran 2005).

Two other organisms commonly associate with aphids. Ladybird beetles are one of the most prevalent and important aphid predators; a single ladybird visit can reduce aphid populations to a third their original size and induce the production of winged offspring (Minoretti & Weisser 2000). Ants also tend many species, protecting aphids from predators in order to harvest the honeydew the aphids excrete. However, the traditionally assumed beneficial nature of this relationship is currently being challenged (Billick *et al*. 2007). Both ants and ladybirds host their own suite of symbionts and other flora (Majerus *et al.* 1999; Boursaux-Eude & Grossb 2000).

Born to Run

The secondary symbionts of aphids provide a fascinating opportunity to explore transmission mechanisms and patterns. The close association of secondary symbionts and host clades, and the readily observable transmission from mother to daughter support a predominance of vertical transmission, but do not tell the whole story. Unlike *Buchnera*, these secondary symbionts are found in various tissues within aphids and are also scattered across populations and taxa (Russell *et al.* 2003). This spotty coverage both illustrates that secondary symbionts are not required and that there must be an additional source of transmission, as new secondary symbionts appear in species whose ancestors did

not appear to possess the symbiont (Tsuchida *et al*. 2006). Lines of *B. aphidicola* show more than twice the divergence from each other than occurs between secondary symbionts, suggesting along with the sporadic prevalence of these symbionts (Tsuchida *et al*. 2002) that vertical transmission is not the only source of spread. As aphid lineages possessing *B. aphidicola* diverged, their obligate symbionts did as well. But if secondary symbionts jumped into an already established new species, it would resemble its relation in the original species far more than two *B. aphidicola* strains that diverged with the two species millions of years previously.

Horizontal transfer, the movement of bacteria to unrelated members of the same species or across taxa, has been suggested as an additional source of symbiont spread (for example see Russell *et al* 2003). Evidence comes in varied forms, from relationships of bacteria across host species to observation of sexual transmission. Whiteflies, for example, possess a symbiont closely related to an aphid symbiont that may have diverged only 17-34 million years ago—over 200 million years after the insects diverged (Darby *et al*. 2001). Unlike aphid's *Buchnera*, which followed a strict pattern of vertical transmission, such a timeline as this divergence between the whitefly symbiont and aphid symbiont strongly supports a bacterial leap across species. Closely related *Spiroplasma* symbionts, with over 98% 16S rDNA similarity to each other, have been found in the insect

orders *Coleoptera* (Majerus *et al*. 1999), *Lepidoptera* (Jiggins *et al*. 2000), *Hemiptera* (Fukatsu *et al*. 2001), and ticks (Weisburg *et al*. 1989). Another recently discovered aphid symbiont is a closely related member of the genus *Rickettsia* (Werren *et al.* 1994), a group known for its predominance of arthropod-vectored vertebrate diseases. Members of this genus infect beetles and ticks as well (Philip *et al*. 1983). This observed diversity in hosts is not uncommon among facultative symbionts; it suggests either an inherent ability of symbionts to live in a diverse array of hosts or else a source of new genes needed for host expansion, such as transfer of plasmids or other mobile DNA units, for which symbionts are well-known (Russell *et al*. 2003; Moran 2006). Symbiont genomes undergo high levels of mutation—up to ten times that of other bacteria—with a penchant for rapid evolution. Facultative genomes are dynamic, containing mobile elements, bacteriophage and evidence for recombination (Moran *et al*. 2009; Moran *et al*. 2008). Such fluid genomes would ease the establishment of new infections in novel hosts (Moran 2006). The degree of similarity among the 16S rDNA of three types of pea aphid symbionts suggest recent moves that are likely still an ongoing process for these bacteria (Russell *et al.* 2003). Other symbionts have demonstrated a wide range of transfer; *Wolbachia pipientis*, a bacterium first characterized in arthropod hosts that infects up to 70% of insect species, is

efficiently transmitted vertically but also exhibits widespread horizontal transfer in field populations even among distantly related hosts (Moran 2006).

Potential routes of horizontal transfer are not clear. In laboratory conditions, secondary symbionts demonstrate vertical transmission in aphids, with no occurrences of horizontal transfer (Sandström *et al*. 2001). However, survival of experimentally-transferred bacteria has been demonstrated; Russell and Moran (2005) injected hemolymph from infected hosts and established new maternal aphid lines that faithfully executed vertical transmission, even with novel symbionts from other host species. Chen and Purcell (1997), among others, hypothesized that aphids could transfer secondary symbionts to one another via host plants, but did not observe any such transmission through the pea plants in their experiment. The similarity of one type of secondary symbiont to a bacterial pathogen of ladybirds, *Chilomenes sexmaculatus* (Werren *et al.* 1994), between ladybird and aphid symbionts, (Majerus *et al.* 1999) and between *W. pipientis* found in whiteflies and the symbionts found in the whitefly's parasites (Darby *et al.* 2003) makes a method of transfer through predator-prey or host-parasite interactions a tempting hypothesis. *H. insecticola* has been detected in aphid honeydew samples as well (Darby & Douglas 2003), suggesting oral transmission as a source both for horizontal transfer across aphid lineages and into other species such as ants that harvest the honeydew. Additionally, Moran and Dunbar

(2006) demonstrated sexual transmission of symbionts into uninfected aphids. Both paternal and maternal symbionts could be passed to both sexually and asexually produced offspring, uninfected females became infected with the male's symbiont after mating, and often co-infections occurred in already infected females but were not sustained (Moran & Dunbar 2006).

The similarities of symbionts associating with related species implies that such transfer is more likely among similar organisms than across more distant relations (Russell *et al*. 2003). Such a finding is supported by the predominance of human diseases originating in apes, our closest relatives, despite a scarcity of interspecies interactions (Wolfe *et al.* 2007). The phylogenetic similarities of symbionts and the plethora of observed vertical transmission against the paucity of observed horizontal transfer events in laboratories imply impediments to horizontal transmission. Such limitations may include limited opportunities for the interactions needed for transfer, difficulty surviving in novel hosts, poor methods of vertical transmission to offspring in novel hosts or even death of the new host from maladaptive effects of the bacterium (Russell *et al.* 2003). For aphid symbionts, horizontally transferred symbionts from one aphid species to another can have difficulty establishing sustained vertical transmission or can induce negative affects such as reduced growth, prolonged development time and susceptibility to heat (Russell & Moran 2005). Such challenges may reduce

the successful occurrence, if not actual occurrence, of horizontal transmission. However, with the maximum failure rate calculated for vertical transmission of *H. insecticola* at 2%, a horizontal transmission rate of 3% would be sufficient to maintain previously documented prevalence rates of 37% (Darby & Douglas 2003).

The study

Through PCR-based surveys of natural populations coupled with controlled laboratory experiments, I explored: 1) the prevalence of common pea aphid secondary symbionts in aphid communities and in associated ladybird beetles and ants; 2) the ability of the aphid symbiont *Serratia symbiotica* to establish infections in ladybird larvae and adults; and, 3) the impact on various predator life characteristics from a diet of aphids possessing symbionts.

METHODS

Aphid community collections

Collections of aphids, ants and ladybird beetles took place between March 2008 and August 2008 in Atlanta, GA and Centennial, Colorado. We took additional collections in Atlanta in April 2009. We gathered aphids from common vetch (*Vicia sativa*), alfalfa (*Medicago sativa*) and clover (*Trifolium repens*). Any ants or ladybird larvae and adults found on the same plant as the aphids were collected as well. All samples were frozen at -20°C after collection. Two species of aphids were collected, *Acyrthosiphon pisum* (pea aphids), and *Aphis craccivora*, (cowpea aphids). Both species share similar plant hosts and insect communities. Individuals of these samples were then extracted and tested for the presence of *H. defensa, R. insecticola* or *S. symbiotica* using the standard PCR assays described below.

Review of prevalence of symbionts in Georgia lines

Fourteen clonal lines of pea aphids were established from aphids collected in Georgia during the community survey. Two sample aphids from each line were extracted and tested for all three symbionts using standard PCR assays described below.

Extractions

All collected and experimental insects underwent full-body extractions following the standard protocol using QIAGEN DNeasy Blood and Tissue Kit. In preparation for extraction, each sample was washed with 500 µL of 99% extraction-quality ethanol, followed by 500 µL of PBS buffer. Each sample was then individually frozen in liquid nitrogen and ground with a disposable plastic pestle. For the first incubation step, ants were incubated overnight (16-20 hours), ladybirds were incubated for 3-4 hours and aphids incubated for 30 minutes. Samples were eluted in 100 µL of elution buffer and stored at 4°C.

PCR reactions

The quality and presence of the DNA samples was confirmed with HCO/LCO primers commonly used to amplify a section of the cytochrome oxidase gene of insects. These reactions used Platimun Taq polymerase from Invitrogen (see appendix 1.a for the protocol). PCRs were then run on all samples that passed quality control to test for the presence of secondary symbionts. These tests used forward primers for the same portion of 16s rDNA but specific to each species of symbiont, with a general reverse primer (see appendix 1.b for protocol). All reactions were visualized on 1.5% agarose gels for presence/

absence of a symbiont-associated band. A positive control (i.e., insect sample known to have target symbiont) was run during all reactions.

Live ladybird experiments

All ladybird beetle experiments were carried out using *Hippodamia convergens* adults (Ward's Natural Science) or their offspring. For the larval comparisons, eggs were collected from adult ladybird pairs fed aphids with no secondary symbionts and allowed to hatch. Pea aphids for feeding were raised in large butterfly cages on fava bean plants (*Vicia faba*).

To explore ecological effects of horizontal transmission, I conducted experiments to see the effects of secondary symbiont ingestion of life-history. I recorded aphids consumed per day, survival rates and egg production in paired adults. Twenty-two pairs of larvae were only fed aphids infected with *Serratia symbiotica* (5AR aphids) and 20 pairs of larvae were only fed aphids without any secondary symbiont (5AO aphids). These clonally identical aphid lines with and without symbionts were previously established for an experiment assaying symbiont-protection against parasitoid wasps (Oliver *et al*. 2005). Each pair of beetle larvae was housed in a 2"-by-2" plastic box (to avoid losing the small larvae on a plant) and fed fresh aphids every 24 hours. All aphids remaining in the box were counted before new aphids were added, and all old aphids were

replaced with fresh aphids. Providing only third instar aphid nymphs controlled for size of aphids. The beetle larvae were first fed only four aphids; this number was increased any time the larvae did eat or nearly ate all aphids present. Due to the delay in hatching, data were collected over 15 to 18 days depending on the pair. Pairs were always exactly the same age.

For the adult mating comparisons, sixteen pairs of ladybugs were placed on individual fava bean plants with vented cup lids. All remaining aphids were counted every 24 hours, and then the total number of aphids was returned to 30 (only fifth instars—those that began to reproduce were removed). Sexing ladybirds is difficult, so the pairs that had not being observed mating were switched after two days, at which point all had been observed to be mating. The number of eggs laid was counted every day for 8 days, noting group size and whether any eggs had hatched or been eaten (ladybird females are known to occasionally eat eggs).

Persistence of S. Symbiotica in ladybird beetles:

Four experiments were constructed to test the extent to which *S. symbiotica* could establish an infection in a new host:

1) Seven beetle larvae raised on 5AR for 18 days were switched to 5AO aphids. These larvae were to be fed 5AO for one week, but at two days only two remained larvae. The larvae were frozen at two days. The pupae were allowed to hatch and then frozen immediately.

2) To explore effects of digestion, 60 ladybird adults kept in large butterfly cages with 5AR aphids for two weeks. The original protocol was to transfer the ladybirds to a cage with 5AO aphids, and then remove 20 ladybirds after 24 hours, 48 hours and 7 days after transferring them. Only 10 ladybirds remained after 2 weeks, so these were transferred to a cage with 5AO aphids and frozen after 48 hours.

3) A test was conducted with a new group of adult ladybirds to see how long ingested *S. symbiotica* lasted. Nineteen ladybirds were fed one or two 5AR aphids and then frozen under one of 5 conditions: instantly, after 1.5 hours, 3 hours, 4 hours or 7 hours.

4) Eleven larvae raised on 5AO aphids for 16 days were starved for 24 hours and then each fed one 5AR aphid. Larvae were frozen under one of 6 conditions: instantly, at 1 hour, 2 hours, 4 hours, 6 hours or 10 hours.

Additionally, all ladybirds that died during the experiment were frozen, as well as any larvae still alive at the conclusion of 18 days.

Statistics

All statistical analyses were conducted using R 2.8.1 for Mac (OS X Tiger). Data were checked for normal distribution of error and homogeneity of variance. The log of aphids eaten by the larvae was used for statistical tests to create linearity and normal distribution. Linear mixed-effects model to account for the repeated measures of the same beetles were fit to the data sets of number of aphids eaten by both ladybird adults and larvae per day, as well as for egg production data. The difference in survival rates was statistically tested using a z-test. The number of aphids eaten for the larvae and for paired adults was averaged for each box according to the number of ladybirds in the box (to account for the death of any larvae).

RESULTS

Aphid Community Collections

Of the 95 successful individual DNA extractions from insect community collections, three aphids showed positive for *H. defensa* while no ants or ladybirds tested positive for any of the three symbionts (*Serretia symbiotica, Regiella insecticola* and *Hamiltonella defensa*). These 95 samples were derived from 40 aphids, 36 ants and 19 ladybirds (Figure 2).

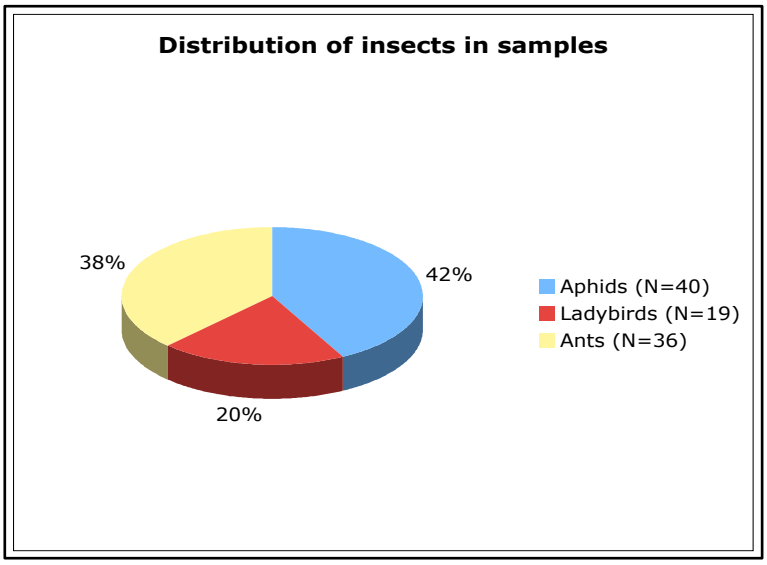

Figure 2: Breakdown of insect types in community collections. Aphids and any ladybirds or ants found with them were collected from Colorado and Georgia during the spring and summer of 2008.

Review of prevalence of symbionts in Georgia lines

All 28 of the Georgia line aphids were successfully extracted. Five lines contained *S. symbiotica*, one line contained *H. defensa* and two lines contained *R. insecticola*. Six lines did not have any of the three secondary symbionts, and no line had a double infection (Figure 3).

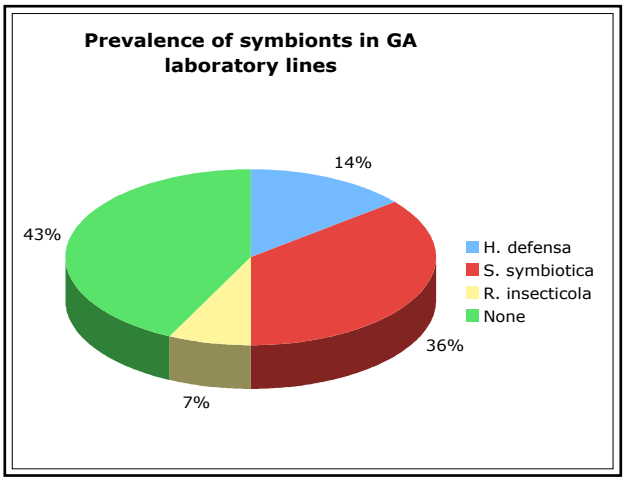

Figure 3: Proportion of symbionts present in laboratory lines. Two aphids from each of the fourteen lines established from field-collected samples were tested for three main secondary symbionts.

Ladybird experiments

Thirty of the original 44 5AR-fed larvae and fifteen of the original 40 5AO-fed larvae remained at the conclusion of the experiment. Larvae fed 5AR aphids had a higher survival rate than larvae fed 5AO aphids (z-test, p=.00493). Two larvae in each sample were observed being cannibalized and one larva in each

sample died during handling; these deaths were excluded from the statistical tests.

The number of aphids eaten by the larvae increased non-linearly over the 18 days (Figure 4). The log of the number of aphids eaten was used to achieve a normal distribution (Figure 5). The number of aphids that 5AR-fed larvae consumed per day was not significantly higher than those larvae eating 5AO, while day did contribute significantly to the variance ($p < .001$). The adult ladybirds demonstrated a similar non-significant difference between treatments, as well as the fact that the number eaten did not vary significantly by day (Figure 6).

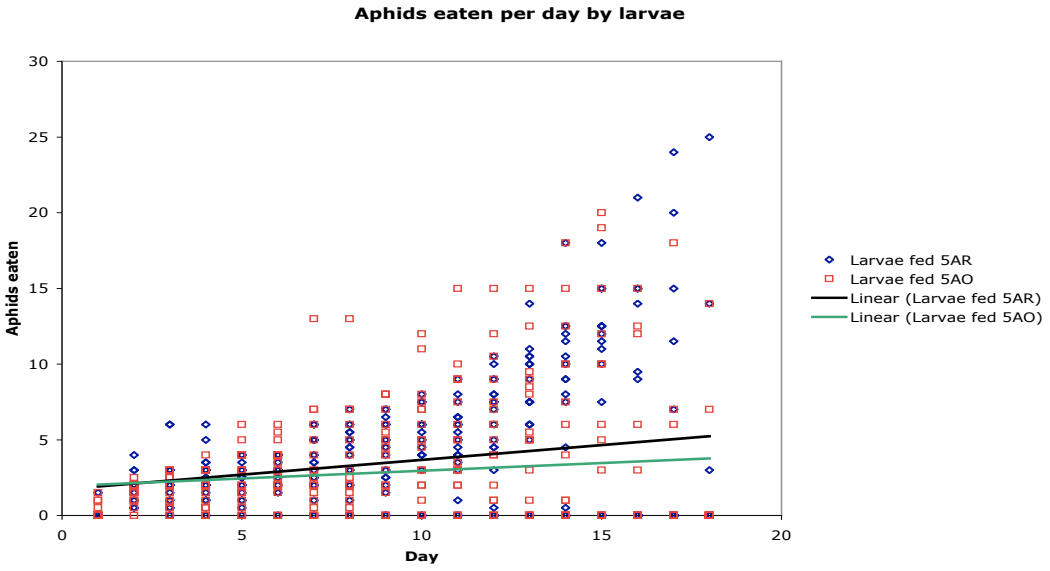

Figure 4: The number of aphids eaten by beetle larvae per day as an average of each box per larvae. There was no significant difference between the treatments.

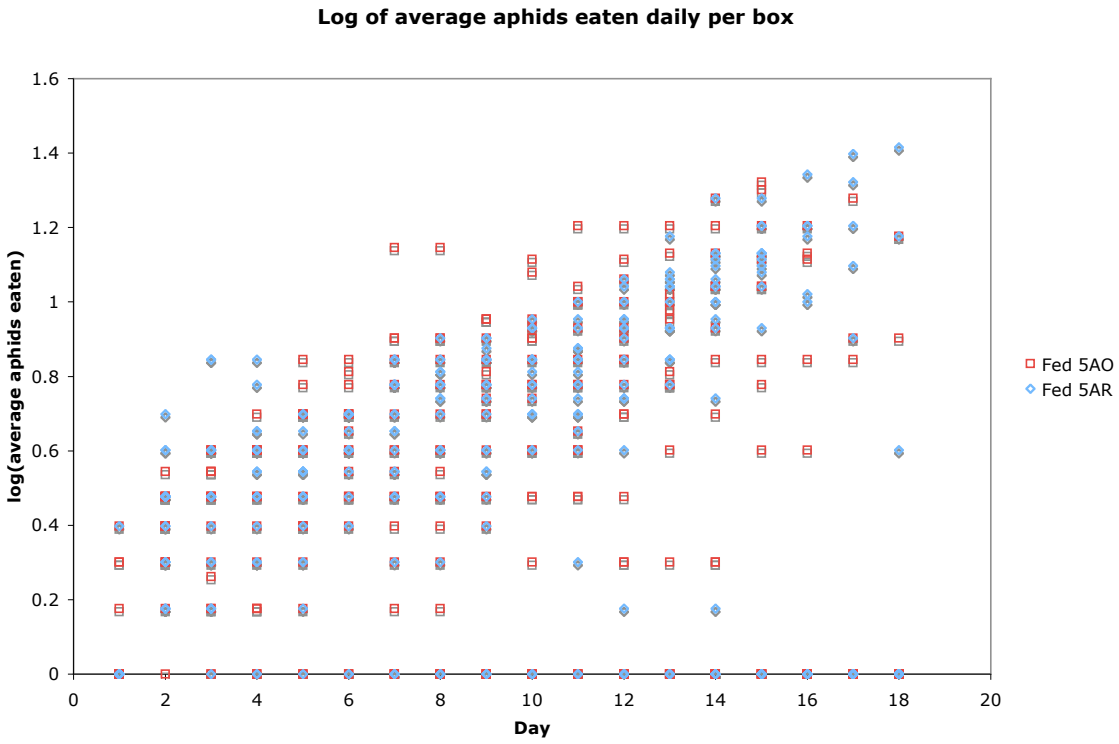

Figure 5: The number of aphids eaten per day as an average per larvae per box. The data showed linearity when I considered the log of the average aphids eaten per larvae box.

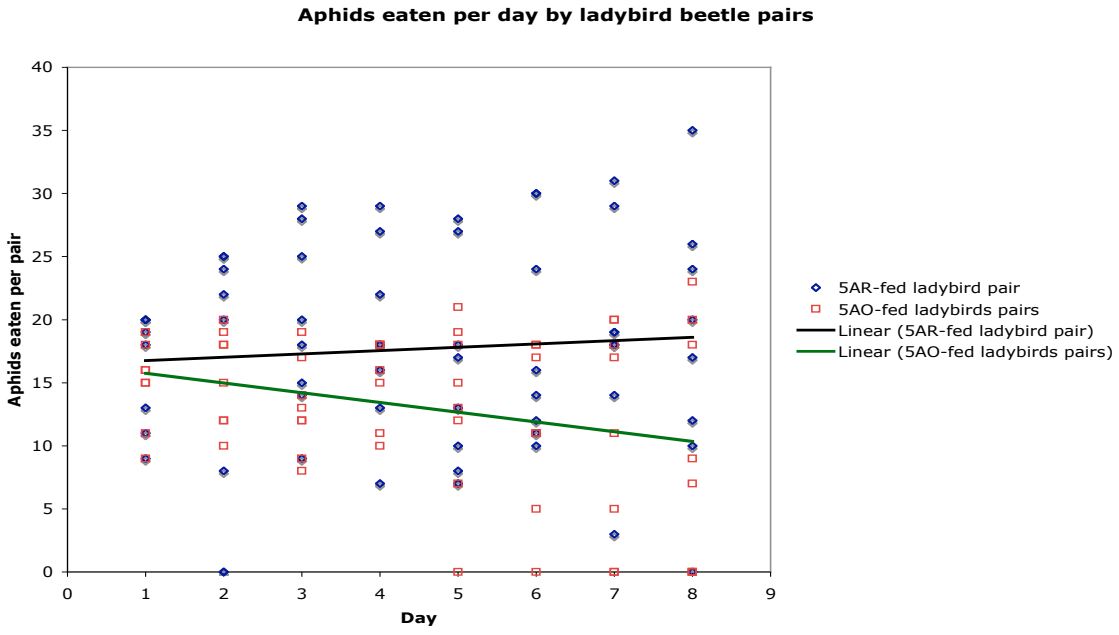

Figure 6: Aphids consumed by adult ladybird beetle pairs did not increase by day or vary significantly by treatment.

All but one of the 21 of the adult ladybirds fed 5AR once passed quality control and tested positive for *S. symbiotica*. The one negative test was a ladybird fed 2 5AR aphids and frozen at 1.5 hours (Figure 8). Fourteen of 17 larvae fed one or two 5AR passed quality control after extraction, and of those 11 tested positive for *S. symbiotica*. One of the two ladybirds larvae each frozen at 6 and 10 hours tested positive (Figure 8).

All but two of the seven larvae raised on 5AR and switched to 5AO pupated. One of the larvae tested positive for *S. symbiotica* while the other did not. One of the two pupating 5AR-fed larvae that passed quality control tested positive for *S. serratia*. Two of the eight adult ladybirds fed 5AR for two weeks and then switched to 5AO for one week tested positive for *S. symbiotica.*
All of the larvae that died during the experiment whose bodies were recoverable (only 10) tested positive for *S. symbiotica*. This included both larvae raised on 5AO and 5AR aphids.

There was no significant difference in the number of eggs present per day across the treatments (Figure 9).

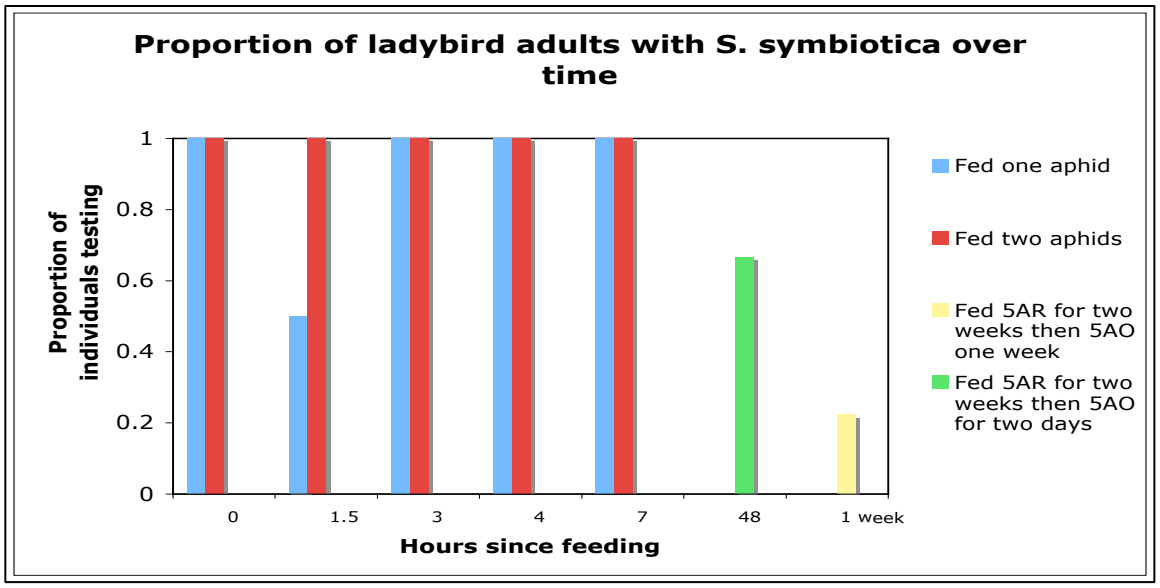

Figure 7: Experimental test of the persistence of *S. symbiotica* in adult beetles. 0-7 hour samples were starved after eating 5 AR aphids. 48 hour and 1 week samples, after final 5AR aphid feeding, had their diet replaced by aphids without *S. symbiotica.*

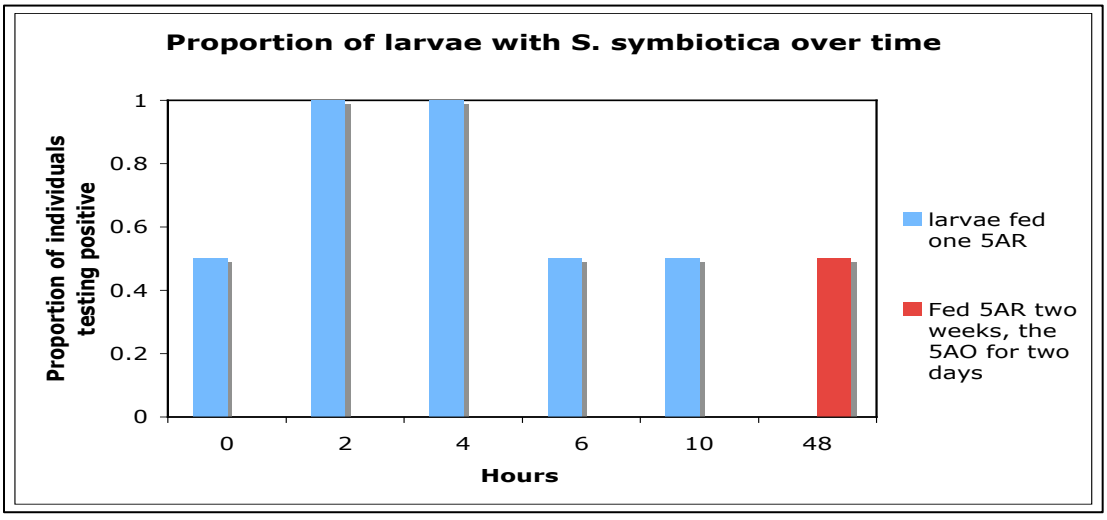

Figure 8: Experimental test of the persistence of *S. symbiotica* in beetle larvae. 0-10 hour samples were starved after eating 5 AR aphids. 48 hour samples, after final 5AR aphid feeding, had their diet replaced by aphids without *S. symbiotica.*

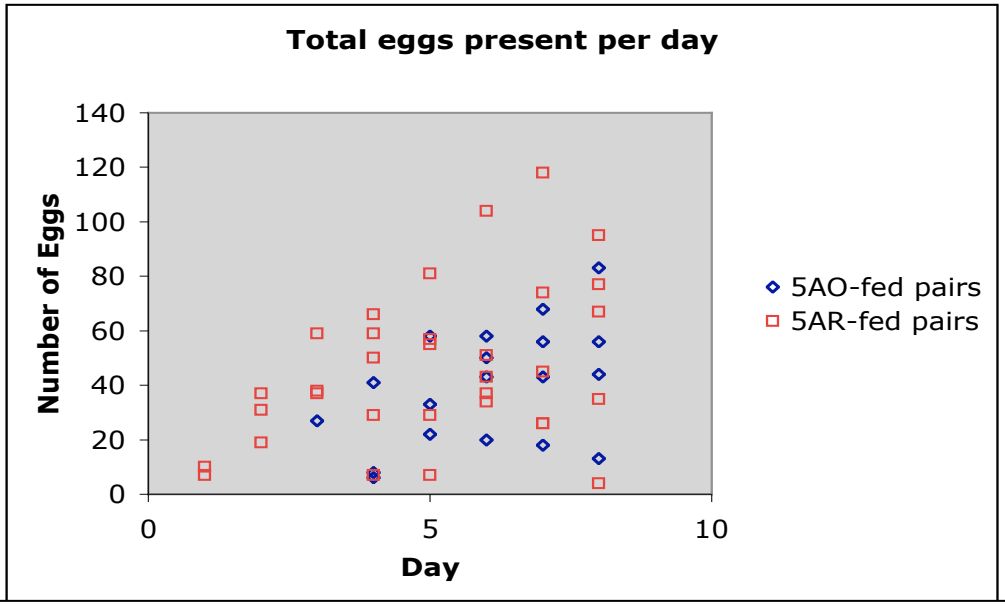

Figure 9: Total accumulation of eggs per ladybird adult pair, counted as how many were present on each day. There was no significant difference between the treatments.

Table 1. Summary of the proportion of symbionts in the various samples.

Sample type	Sample size	Proportion testing positive for *S. symbiotica*	Proportion testing positive for *H. defensa*	Proportion testing positive for *R. insecticola*	Proportion with no symbiont
Community screen	95	0.00	0.03	0.00	0.97
Ants	36	0.00	0.00	0.00	0.00
Aphids	40	0.00	0.08	0.00	0.93
Ladybirds	19	0.00	0.00	0.00	0.00
Georgia lines	28	0.36	0.07	0.14	0.43
Larvae fed 5AR two weeks, then 5AO two days	7	0.50	NA	NA	0.50
Larvae fed one 5AR then frozen over time	14	0.79	NA	NA	0.21
Ladybirds fed one 5AR then frozen over time	21	0.95	NA	NA	0.05

DISCUSSION

Our knowledge of bacterial diversity is extremely limited; ten years ago, only 5000 non-eukaryotic organisms had been formally described in contrast to the half-million described insect species (Pace 1997). Such a discrepancy adds to the fog surrounding the complex interactions of communities and their microbiota. Vertically transmitted symbionts occur commonly across the insect world, and yet in many cases the role of the symbiont remains unknown (Oliver 2005). The relationship of aphids and their symbionts is one of the few systems particularly well examined (Tsuchida 2006). Yet, while some studies have explored symbiont-conferred benefits and the potential to establish symbionts in new insect hosts, the vast majority of studies on consuming beneficial symbionts have concerned humans and animals of agricultural importance (Salonen *et al*. 2009). Comparisons from vertebrates, especially mammals, to insects must be viewed with extreme caution, but may still provide some useful insights.

The low occurrence of secondary symbionts in the collections in this study did not coincide with published findings for either species of aphid or the experimental finding in the Georgia lines, while the finding in the lab-maintained Georgia lines more consistently reflected other prevalence studies. Pea aphids have been found in published studies to harbor all three types, as well as two other secondary symbionts, in a relatively high occurrence; 54.7% of pea aphids

surveyed in Japan contained either *S. symbiotica* or *R. insecticola* (Tsuchida *et al.* 2002). The higher rate of *S. symbiotica* in the Georgia lines reflected the prevalence found in other studies of aphids in the field; *S. symbiotica* was the most common in three studies, occurring in 87.7% of clones collected in California (Chen & Purcell 1997), in 50.8% of clones collected in Japan (Fukatsu et al., 2001) and in 37.5% in a sample of US clones (Sandström *et al.* 2001). *R. insecticola* was less common, occurring in only 22.2% of samples (Sandström *et al.* 2001). Only 33.6% of samples in one study of Japanese pea aphids did not have a secondary symbiont (Tsuchida *et al*. 2002). While 43% (n=14) of laboratory lines, 93% of the community screen did not possess any other the three main symbionts. I cannot explain the difference between infection percentages for naturally occurring aphids and laboratory lines, as both sets of aphids were collected at the same time and the same Georgia localities. The question arises whether or not laboratory living could select for symbiont presence; several lines died after collection from fungal infections, against which symbionts are known to provide protection. This lack of secondaries could also potentially be indicative of changes occurring within Georgia and Colorado populations. However, the finding could also be due to an error in processing or artifact of sampling in light of other prevalence studies. Possible errors sources in this finding are discussed with other potential errors later.

The paucity of these three symbionts occurring in ants or ladybirds from the community collections is consistent with the general thinking that straight horizontal transmission is a relatively rare event (Russell *et al.* 2003). *Tsuchida et al*. (2002) looked for *Wolbachia* and *Arsenophonus*, symbionts commonly found in various insects, and found no evidence in over a thousand collected aphids in Japan. However, it is difficult to consider these data as confirmative evidence, as there were so few aphids with secondary symbionts. A sample of three gives hardly any information about whether ants and ladybirds in the field pick up the symbionts of sympatric aphids. Additionally, broader bacterial screens probably would have been more informative, as the bacterial colonization of insects is likely far broader than the characterizations currently available (Moran & Dunbar 2006); perhaps other (or even undiscovered) symbionts or at least commensal species are shared across communities.

The difference between the survival rates of the two larval groups suggests a benefit of some kind was conferred to larvae consuming symbiont-containing 5AR aphids when compared to eating symbiont-free 5AO, even if there was inconsistent evidence for any kind of sustained colonization. Larvae eating 5AO aphids were more than twice as likely to die than larvae eating 5AR aphids. This survival differential could have resulted from protection from pathogens by ingesting the symbiont, supplementation of crucial nutrients, both

or even an entirely different mechanism. Increased resistance against pathogen colonization occurs in aphids possessing secondary symbionts. *H. defensa* protects aphids against parasitoid wasp eggs (Oliver *et al.* 2008) as well as fungal pathogens (Scarborough *et al*. 2005), and *S. symbiotica* confers resistance to the parasitic wasp *Aphidius ervi* (Oliver *et al.* 2006). While these effects originate in symbionts with established infections, benefits from merely consuming symbionts has been observed in chickens: USDA developed a product called PREEMPT, a blend of 29 different intestinal bacteria from chickens, that was shown to effectively protect against *Salmonella*, *E. coli* O157:H7, *Campylobacter*, and *Listeria* colonization (USDA Press Release 0122.98, March 19, 1998). The bacteria do not necessarily need to originate from the same host species either; human probiotic bacteria can increase feed conversion efficiency in piglets, measured as kg of food intake/kg of weight gain (Matijasic *et al.* 2004), so it is conceivable for ladybirds to receive benefits from aphid symbionts.

Mere exposure to a constant source of bacteria may have increased survival of symbiont-inoculated beetles. Germ-free animals are more susceptible to infection (Tannock, 1998); exposure to environmental microbes in young vertebrates (e.g. mice, humans) has been correlated with increased immunity and decreased allergies later in life and resistance to parasites has been correlated with persistent immune challenges (Yazdanbakhsh *et al.* 2002). Studies in insects

also have shown that exposure to an initial bacterial infection can decrease susceptibility to a second infection, and thus the symbionts may serve to prime insect immune responses as well (Roth *et al*. 2009).

Symbionts offer many species additional nutrients, from aphids to humans (Moran 2006; Sanders 2000). The larvae may be able to directly use amino acids or other beneficial compounds produced by the symbiont or may extract such resources from the aphid that the symbiont produced before the aphid was consumed.

That *S. symbiotica* did not persistently infect the ladybirds does not preclude the beetles from deriving advantages. Establishment of a sustained colonization is not necessary to gain benefits, nor is it commonly found in probiotic studies; after two weeks of humans ingesting the symbiotic bacterium *Lactobacillus rhamnosus* GG, fecal cultures showed a marked decline in the presence of the probiotic after treatment stopped (Alander *et al.* 1999). In fact, some benefits from probiotics are not dependent on viable cells, such as reduction of hypertension resulting from the fermentation end-product in a food product (Sanders 2000).

It is not surprising that number of aphids consumed increased by day for the larvae, as they were clearly growing. Likewise, it is expected that the adult

ladybirds did not show significant increase over the days, as they were not growing.

Several possible sources of error should be considered. First, the quality of the aphids may have differed by more than their symbiotic content; fluctuations in population density in the cages, the quality of the plants and other environmental factors could have impacted aphid health (Dixon 1977) and thus inadvertently affected the ladybirds that ate them. Second, the result that ladybirds never intentionally exposed to *S. symbiotica* tested positive for it suggests either contamination in feeding or extractions, or a false positive from a similar bacterium. The R1279f primer is known to occasionally react with the DNA of other bacteria, and *S. symbiotica* is very similar to many other free and commensal species (Russell *et al*. 2003). This should, however, have generated a different banding pattern on the agarose gel, and the positive bands always appeared similar to the positive control. Third, the symbiont distribution in the field may vary by region (Tsuchida *et al.* 2002); as collections occurred in narrow regions in Atlanta and Centennial, this variation may have impacted which symbionts were—and were not—observed. One aphid symbiont has demonstrated a geographical cline on the main island of Japan (Tsuchida *et al.* 2002), suggesting that climate and latitude may play a role in symbiont distribution; if this is the case, the differing locations and climates of Georgia and

Colorado could have contributed to different findings. Fourth, the unexplained death and disappearance of such a large number of adult ladybirds in the sustained-infection experiments suggests either that the system was not completely closed (and thus ladybirds could escaped somehow) or that something was misaligned (such as fungal growth) within the cages. I found very few ladybird bodies outside the cages, although on two accounts I saw and caught a loose, live ladybird within the ladybird room. I also never found any bodies outside the room in the greenhouse. All of these factors should be accounted for in future experiments. Lastly, the quality of the bacterial DNA could have been affected even thorugh the insect DNA passed the quality control PCR, or could have been in abnormally low quantities. A follow-up test for *Buchnera*, which all the aphids should have, could show whether or not this is the case.

FUTURE DIRECTIONS AND CONCLUDING REMARKS:

The differences observed in aphids consumed and in the survival rates of larvae between the treatments needs to be repeated; in such a complex system, the interplay of numerous and unidentified factors can significantly impact results. If the findings within this study stand, then it suggests new questions previously unexplored in the insect world. If predators can actually derive a benefit from consuming symbionts even if there is no colonization, could this impact the prevalence of symbionts in prey species? Is there a difference in benefit conferred based on the species of bacterium or the organism consuming the symbiont? Does eating symbionts as a larvae increase survival as an adult, even if consumption ceases? One possible test would be a longer experiment that follows ladybird larvae all the way through pupation and through their adult lives.

Is ingestion actually a mechanism for establishing infections in novel hosts? Although we did not observe any sustained infection in adult ladybirds, our sample size was unfortunately small. Perhaps a larger sample would demonstrate variability in length of persistence, which would be consistent with the fact that success of infections is dependent on host and symbiont factors (Moran & Dunbar 2006). If so, it is conceivable that if ingestion is a feasible

mechanism for delivering viable symbionts, then the right bacterium could meet up with the right host to establish a firm colonization.

The impact of symbionts on ecological processes and evolutionary history is starting to emerge: as Moran (2006) explained, "It is now clear that symbioses have been crucial in adaptive radiation, lineage evolution, and ecological diversification." Understanding symbiotic transmission greatly contributes to this growing comprehension, and can even apply to agricultural solutions. Secondary symbionts modified to prevent transmission are being considered for biological control pathogens of aphids, tsetse, bedbugs and other damaging insects (Darby *et al*. 2003); but we should understand the chance of horizontal spread to other taxa or genetic recombination with temporary insect microbiota guests before attempting any implementation.

A more complete understanding of bacterial ecology and transfer within communities is critical not just in the insect world. Bacteria have accompanied man as he first stood up from his ape ancestors. Many species he brought with him, as bacteria often evolve in tight association with their hosts (Brunham 1993). Yet many of the pathogens that plagued man were zoonotic in origin, hopping into humans from prey and other animals with which early man collided. The importance of this source of disease is only recently being truly appreciated. Not only have historically important bacterial diseases like diphtheria, measles,

smallpox and tuberculosis originated in domestic livestock (Wolfe *et al.* 2007) but the WHO in 2009 asserted that nearly 75% of emerging diseases in the past decade alone are zoonotic. In a recent review of risk factors for emerging diseases, a zoonotic species was twice as likely to be associated with emerging disease as a non-zoonotic species (Taylor *et al*. 2001). With such threats as the avian flu standing only mutations away from human-to-human transmission and a possible pandemic (Russell & Webster 2005) and zoonotic agents like anthrax growing as bioterrorism weapons, the importance of understanding how bacteria move among species and across groups will only increase.

APPENDIX A:

Protocol for "hco/lco" primer DNA quality control test
(Adapted from CCDB)

For one reaction, mix in the following order:

PCR-quality water	1.7525 µL
10% Trehalose	6.25 µL
10x buffer	1.25 µL
50 mM $MgCl_2$	0.625 µL
10 mM dNTPs	0.0625 µL
5 µM primer (forward)	0.250 µL
5 µM primer (reverse)	0.250 µL
Platinum Taq	0.06 µL
Total (per rxn)	10 µL

Aliquot the master mix into PCR tubes or plates. Add 2 µL of the DNA sample for a total reaction mix of 12.5 µL.

APPENDIX B:

Protocol for bacterial test PCR

For one reaction, mix in the following order:

PCR-quality water	16.85 µL
AmpliTaq Buffer	3 µL
10 mM dNTPs	1 µL
Primer (forward)	3 µL
Primer (reverse)	3 µL
AmpliTaq	0.15 µL
Total (per rxn)	27 µL

Aliquot the master mix into PCR tubes or plates. Add 3 µL of the DNA sample for a total reaction mix of 30 µL.

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