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LETTER

Interspecific transmission of a male-killing bacterium on an ecological timescale

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Abstract

Inherited symbionts are important drivers of arthropod evolutionary ecology, with microbes acting both as partners that contribute to host adaptation, and as subtle parasites that drive host evolution. New symbioses are most commonly formed through lateral transfer, where a microbial symbiont passes infectiously from one host species to another, and then spreads through its new host population. However, the rate of horizontal transfer has been regarded as sufficiently low that population and coevolutionary processes can be approximated to one, where the symbiont interacts with a single host species. In this paper, we demonstrate experimentally that horizontal transfer of the son-killer infection of *Nasonia* wasps occurs readily following multi-parasitism events (two species of parasitoid wasp sharing a fly pupal host), and provide phylogenetic evidence of recent and likely ongoing transmission amongst members of the community of wasps utilizing filth flies. Combining per contact transmission rates estimated in the laboratory with rates of multiparasitism in the field produces an estimate that an infected *Nasonia vitripennis* individual in an Eastern US bird's nest habitat has a 12% chance of passing the infection into *N. giraulti*. We conclude that the single host-single symbiont framework is therefore insufficient for understanding the population and evolutionary dynamics in this system and caution against blind acceptance of the single host/single symbiont framework. We conjecture that lateral transfer rates that require a multi-host framework will most likely be seen in symbionts that retain the ability to cross host epithelia, and that this will be correlated to the recency with which the symbionts have been free living.

Keywords

Arsenophonus, arthropod, male killing, reproductive parasite, symbiosis.

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INTRODUCTION

The last 30 years has seen a revolution in entomology, with the appreciation of the importance that symbiotic relationships with microorganisms play in the life of arthropods. The interactions observed vary from obligate requirements for symbiont presence (providing anabolic function; Dadd 1985; Douglas & Prosser 1992), through ecologically contingent advantages of symbiosis (such as natural enemy resistance; Haine 2008), to interactions with symbiotic microbes showing very subtle manipulations of host reproduction that promote their own spread, and may, through their parasitic phenotypes, themselves drive host ecology and evolution (Werren *et al.* 2008; Engelstadter & Hurst 2009).

The case studies above have demonstrated the evolutionary and ecological importance of symbiosis in particular host species. In most cases, the symbiont becomes part of the 'extended genome' of the host, being transferred vertically from a female host to her progeny (Moran *et al.* 2008). However, most symbioses originate following lateral transfer of an existing symbiont from one host species to another. Phylogenetic evidence indicates the secondary symbionts of aphids, which provide defense against natural enemies, commonly transfer between species (Russell *et al.* 2003; Moran & Dunbar 2006). Likewise, *Wolbachia*, most commonly a 'reproductive parasite' of arthropods, has an extensive history of movement between species (Baldo *et al.* 2006; Raychoudhury *et al.* 2009; Russell *et al.* 2009).

That interspecific horizontal transfer of symbionts occurs, and is important in establishing new symbioses, is beyond doubt (Moran *et al.* 2008). Phylogenetic evidence from infection relatedness in defined communities indicates how ecological bridges such as predation and parasitism may result in transfer of infection (Vavre *et al.* 1999; Noda *et al.* 2001; Dedeine *et al.* 2005), and experiments have verified that symbiont transfer occurs following intimate ecological contact – that is to say, contact where an uninfected host species is exposed to the body fluids of an infected one (Rigaud & Juchault 1995; Heath *et al.* 1999; Huigens *et al.* 2000, 2004; Oliver *et al.* 2003; Scarborough *et al.* 2005; Jaenike *et al.* 2007). However, whilst it is clear that these methods of transmission will create new infections, the likely rate of transfer in the systems under study (measured as the product of the rate at which exposure events occur, and the rate at which these events create a persistent infection in the new species) is very low. Thus, it has been accepted that models of symbiont population biology require only vertical transmission to be considered (Engelstadter & Hurst 2009), and models of coevolutionary dynamics can satisfactorily regarded within a framework where a symbiont is found in a single host species (e.g. Randerson *et al.* 2000; Hornett *et al.* 2010). Lateral transfer is important only in determining the rate and pattern with which new symbioses are initiated (e.g. Engelstadter & Hurst 2006).

As its original description, the parasitic son-killer bacterium *Arsenophonus nasoniae* has been known to transfer between matrilines of its parasitoid host, *Nasonia vitripennis*, when two female wasps utilize the same host (Huger *et al.* 1985; Skinner 1985), a scenario known to be common in nature (Grillenberger *et al.* 2008). Thus, in contrast to other reproductive parasite infections, infectious transmission within the species must be taken into account in the population biology of the infection. A key question with regard to the coevolutionary dynamics of the system is the extent to which this mechanism of intraspecific transmission also effects symbiont transfer between species. For *N. vitripennis*, multiparasitism, where a single pupal host is utilized by heterospecific parasitic wasps, is also common in nature, and creates an opportunity for interspecific transmission of infection (Klunker 1994; Balas *et al.* 1996; Grillenberger *et al.* 2009). DNA hybridization assays have also suggested the presence of similar infections in *Nasonia longicornis* (Balas *et al.* 1996). We therefore tested whether multiparasitism efficiently transmitted infection between members of the parasitic wasp community that share filth fly hosts, and examined evidence for this transfer process having occurred in the natural community. We conclude from experimental studies passing infection easily between species following host sharing, and from the close relatedness of *A. nasoniae* strains in multiple members of the filth fly community, that multiparasitism is a very potent means of interspecific transmission of the selfish trait 'son-killer'. Thus, the assumption that symbiont/host population and evolutionary ecology can be approximated to a single host/single symbiont model should be treated with caution.

MATERIAL AND METHODS

Transfer experiments

Arsenophonus nasoniae infected *N. vitripennis* (Hymenoptera: Pteromalidae) was obtained following the method of (Balas *et al.* 1996) by screening wild caught wasps derived from naturally occurring fly pupae in New York State (USA, 2006). Two *N. vitripennis* lines, C49f and C49g, obtained from a single bird's nest, were initially identified as infected by PCR screen, and subsequently characterized as possessing the son-killer phenotype. Infection of C49 lines was then maintained in the lab via selection for female bias each generation coupled with PCR assays for infection. In routine screens for infection, DNA quality was systematically tested by PCR amplification of a conserved region of the arthropod *18S rRNA*, and samples then tested for *Arsenophonus* presence using a PCR assay based on the *16S rRNA* gene (Duron *et al.* 2008).

The following lines were used for experiments involving transfer of infection on multiparasitism: *N. vitripennis* line STDR (with a distinctive scarlet eyed phenotype compared to the brown eyed wild type of the C49 lines), *N. giraulti* line RV2 and *N. longicornis* line IV7 (all lab-bred stocks held by the J.H. Werren lab Rochester, USA), *Melittobia acasta* (Eulophidae) line (S. West lab Edinburgh, UK), *Muscidifurax raptorellus* (Pteromalidae) line (Koppert Biological Systems). Wasps were drawn from laboratory strains maintained by allowing females from each generation to freely parasitize 15–20 fly pupae. With the exception of the C49 lines, all the wasp strains were initially free of *Arsenophonus* infection as indicated by repeated PCR assays. All maintenance and experiments employed the filth fly *Sarcophaga bullata* (Sarcophagidae) as hosts and were carried out under 12 : 12 h light: dark, at 25 °C ± 1 °C. PCR assays of a random sample of twenty *S. bullata* individuals indicated they were uninfected by *Arsenophonus*.

To test the ability of *A. nasoniae* to transfer following multiparasitism exposure, we initially transferred the infection from the wild C49 line into the line STDR, which carried a red eye genetic marker, through superparasitism, following the method of Skinner (1985). Following this, we followed the protocol of Skinner to test whether transmission occurred on multiparasitism as well. To this end, a single mated and infected donor STDR female was allowed to co-parasitize with a single mated recipient 'target' female of another species, in a vial with one fly host and allowed to

lay for 48 h. In addition, single uninfected STDR virgin females were allowed to co-parasitize with single mated STDR females to permit the assessment of the possibility of any false-positives from *Arsenophonus* strains transferred through the environment rather than through infected donor individuals. Fly hosts were opened prior to wasp emergence and recipient wasp offspring separated from donor offspring by eye colour. Recipient (non-red eye) F1 wasps were then allowed to emerge, mate and females were then individually permitted to lay on fly hosts. F2 offspring were similarly allowed to emerge, mate and lay in three fly pupae. DNA was extracted from F0 to F2 parents for PCR assays to verify the presence/absence of infection. Infected lines of each species were then characterized at the F3 with respect to the sex ratio produced by a single foundress, and this was compared to uninfected females from the same line. To estimate the rate of male survival more accurately, virgin infected females were isolated and allowed to oviposit. These produce all male broods, and comparison of the family size to that produced by uninfected virgin females from the same line estimates the effect of infection on the rate of male survival. Further generations were screened via PCR to demonstrate reliable vertical transmission.

Screen for *Arsenophonus* in the filth fly community

Material for a screen of *Arsenophonus* infection, specimens of parasitoids and flies, were collected from various sites in USA (2006), Canada (1997–2003) and in the Western Mediterranean area (2003–2005; listed in Table S1). Freeze-killed sentinel house fly pupae and naturally occurring fly pupae were used to survey the local parasitoid fauna (for more details of the protocol, see Gibson & Floate 2004). To minimize the risk of pseudo-replicating through sampling siblings, we checked infection in only one emerged parasitoid female per host fly pupae.

In screens of both wasp and fly material, DNA quality was systematically tested by PCR amplification of a conserved region of the arthropod *18S rRNA*. Samples were then tested for *Arsenophonus* presence using a PCR assay based on the *16S rRNA* gene (Duron *et al.* 2008). Using a draft sequence of the *A. nasoniae* genome created by 454 sequencing (Darby *et al.* 2010), *Arsenophonus*-specific primers were designed for three protein coding genes, which were then used for multi locus profiling (*fbaA*, *ftsK* and *yaeT*). These genes are found in a single copy in the *Arsenophonus* genome, are likely to be distant within the genome as they were found on different genomic scaffolds and are conserved at the protein level throughout 20 other Enterobacteriaceae. The sequence of fragments of COI (cytochrome oxidase-I) mitochondrial and *18S rRNA* genes of a fraction of infected host specimens were also obtained following PCR amplification. Gene features and primers are listed in Table S2. All the PCR amplifications were performed under the following conditions: initial denaturation at 95 °C for 2 min, 30 cycles of denaturation (94 °C, 30 s), annealing (50–52 °C, depending on primers, 30 s, see Table S2), extension (72 °C, 1 min–1 min 30 s) and a final extension at 72 °C for 5 min. Sequences are deposited in Genbank (accession numbers GU226783–GU226823).

Ten additional *Arsenophonus* strains from various insect species, preliminary characterized by their *16S rRNA* sequences, were also used to analyse the relatedness of *Arsenophonus* strains in different host communities.

Phylogenetic analyses

The *fbaA*, *ftsK* and *yaeT* genes fulfilled all the requirements for reliable indicators of genetic relationships between bacterial strains: the three genes were observed: (1) to be representative of bacterial genes, with G+C content within the range seen in other arthropod-associated bacteria, (2) to be subject to stabilizing selection rather than positive selection substitutions ($K_a/K_s<<1$) and (3) to show no evidence of intragenic recombination events (Sawyer's test *P*-value > 0.18; Table S3). Phylogenetic relationships were then evaluated for *Arsenophonus* sequences of *fbaA*, *ftsK* and *yaeT* genes from 14 different arthropod hosts, as well as sequences of 11 other Enterobacteriaceae members. The evolutionary model most closely fitting to the data, the General Time Reversible model with Invariant sites and Gamma distributed rate variation (GTR + I + G), was determined using hierarchical likelihood ratio tests and Akaike information criterion as implemented on MODELTEST version 3.7 (Posada & Crandall 1998). To analyse phylogenetic relationships, two different methods were used, maximum likelihood (ML) estimation and Bayesian inferences (BI). ML analyses were conducted using PAUP version 4.0 (Swofford 2002). Model parameters were first estimated by ML on a neighbour-joining topology, and were further used in optimal tree searches, which consisted of heuristic searches with Tree-Bisection-Reconnection (TBR) branch swapping. Clade robustness was assessed by bootstrap analysis using 500 replicates. Mr. Bayes v 3.1.2 (Ronquist & Huelsenbeck 2003) was also used to construct phylogenetic trees through BI. For each analysis, the Markov chains were run for 500 000 generations. Bayesian posterior probabilities were obtained from the 50% majority-rule consensus of the tree sampled after the initial burn-in period as determined by checking the convergence of likelihood values across generations. The phylogenetic trees obtained for each of the three protein coding genes and with the two methods were all identical, and we conducted analyses using the 1577 bp concatenated *fbaA*, *ftsK* and *yaeT* sequences.

RESULTS

Experimental interspecific transfer of infection following multiparasitism

We analysed experimentally the ease of transmission of the bacterium following host sharing in multiparasitism. We obtained an *Arsenophonus* positive strain of *N. vitripennis* from a recent field collection, and verified that this was maternally inherited and acted as a male killer. We first transferred the *Arsenophonus* infection from the C49 *N. vitripennis* lines into the STDR *N. vitripennis* line (with a distinctive scarlet eyed phenotype) by co-parasitism, in which maternal inheritance of infection was observed (Table 1). We then used this morphologically distinct STDR infected line as a donor to test transfer rates into uninfected laboratory lines of three species of Pteromalidae, *N. giraulti*, *N. longicornis* and *M. raptorellus*, and a more distantly related species, the eulophid, *Me. acasta*.

We found clear evidence that exposure to an infected individual of a different species during multiparasitism resulted in the transfer of *A. nasoniae* between species. Horizontal transfer was taken as proven when F2 wasps of the recipient line emerged infected. Depending on the recipient species, 75–100% of the lines acquired an infection inside the host pupae, as evidenced by infection being present in F1 individuals. In each case, where the brood was infected, the bacterium was subsequently transmitted to the next generation (Table 1). That vertical transmission was not only present, but also efficient, was attested by *Arsenophonus* presence in > 80% of F2 progeny from infected mothers in each species (Table 1), and in at least one line of all species five generations after transfer (Table S4). In contrast to the above, no occurrence of *Arsenophonus* infection was observed in recipient species in multiparasitism control assays using uninfected donor STDR females ($n = 25$, data not shown). Thus, we can conclude that the *Arsenophonus* infection seen in assays with infected donor STDR females were derived from these donor individuals and not from environmental contaminants.

We then examined whether the *Arsenophonus* infection retained its 'reproductive parasite' phenotype (death of sons) in the species into which it had transferred. For haplodiploid species with labile sex ratio, the rate of male killing is best estimated by comparing family sizes from virgin infected and uninfected females, as here only males are produced. Virgin females from the newly infected lines of *N. vitripennis*, *N. giraulti*, *N. longicornis* and *M. raptorellus* produced significantly fewer sons than uninfected comparators (Mann–Whitney two-sided test, all $P < 0.01$). *Arsenophonus nasoniae* infection was associated with the death of between 50 and 90% of sons, with the highest male mortality seen in the three species of *Nasonia*, and lower mortality in *M. raptorellus* (Table 2: note, this experiment could not be conducted with *Me. acasta*, see below). A similar association was observed within families issued from mated females, with significant sex bias towards daughters observed in the newly infected lines. Sex ratio distortion activity is much less profound in the eulophid species *Me. acasta*. Using fertilized females, infection by *A. nasoniae* was associated with a statistically significant reduction in the number of sons produced, but the rate of male death was of small magnitude, with fewer than 10% of males dying. However, this datum is of poor accuracy, because this species is known to naturally display an extreme female-biased sex ratio with typically fewer than 5% males (Matthews *et al.* 2009).

Having established that *A. nasoniae* could transfer from *N. vitripennis* into other species, we then tested whether transfer back to the original host was possible. Attempts to transfer from transinfected *M. raptorellus* strain to the uninfected STDR *N. vitripennis* strain through multiparasitism were successful in 14 encounters of 16, demonstrating that *Arsenophonus* can move bidirectionally between unrelated species under favourable ecological conditions.

Table 1 Horizontal transmission of *Arsophonus* between species following multiparasitism. *Arsenophonus* infected donor wasps marked +. Recipient wasps were in all cases uninfected with *Arsenophonus* (marked −)

Recipient wasp	Donor wasp	Recipient broods with at least one infected F1 individual (total broods tested)	Broods from infected F1 parents with at least one infected F2 individual (total broods tested)	Frequency of female F2 wasps infected (number tested)
Nasonia vitripennis STDR (−)	*N. vitripennis* C49 (+)	1 (1)	1 (1)	1 (12)
N. giraulti (−)	*N. vitripennis* STDR (+)	15 (15)	5 (5)	0.86 (82)
Nasonia longicornis (−)	*N. vitripennis* STDR (+)	11 (11)	4 (4)	0.79 (19)
Muscidifurax raptorellus (−)	*N. vitripennis* STDR (+)	12 (14)	6 (6)	0.95 (39)
Mellitobia acasta (−)	*N. vitripennis* STDR (+)	9 (12)	7 (7)	0.81 (36)

Results of PCR assays for infection are given as number positive with number tested in parenthesis, save for transmission efficiency, which is given as a proportion daughters infected with number tested in parenthesis.

Table 2 Sex ratio and estimated male mortality for *Arsenophonus nasoniae* infected (+) and uninfected (−) wasps of different species (number of crosses from which mean and error estimated given in parentheses)

		Mated cross			Virgin lay	
Species	Infection	Daughters	Sons	Sex ratio *P* (male)	Sons	Estimated mortality
Nasonia vitripennis C49	−	38.5 ± 3.13 (46)	22.5 ± 2.94 (56)	0.370		
	+	44.1 ± 2.14 (46)	10.4 ± 1.25 (56)	0.19***		
N. vitripennis STDR	−	48.5 ± 3.2 (46)	13.7 ± 1.47 (46)	0.208	52.5 ± 4.57 (27)	0.812
	+	36.0 ± 3.1 (49)	1.7 ± 0.49 (49)	0.06***	9.88 ± 2.23 (17) ***	
N. giraulti	−	42.85 ± 3.6 (26)	4.8 ± 0.4 (26)	0.11	52.8 ± 4.04 (19)	0.893
	+	25.0 ± 2.4 (48)	1.2 ± 0.18 (48)	0.05***	5.64 ± 0.75 (17) ***	
Nasonia longicornis	−	43.6 ± 3.34	3.88 ± 0.38	0.08	51.9 ± 4.22 (16)	0.925
	+	28.5 ± 2.75	1.2 ± 0.17	0.05***	3.9 ± 0.84 (11) ***	
Muscidifurax raptorellus	−	19.4 ± 1.24 (42)	9.1 ± 0.76 (42)	0.32	27.6 ± 2.02 (20)	0.50
	+	21.3 ± 1.94 (37)	4.8 ± 0.78 (37)	0.16 ***	13.7 ± 1.29 (22)***	
Melittobia acasta	−	112.1 ± 4.13 (30)	3.0 ± 0.26 (30)	0.03	NA	NA
	+	107.5 ± 5.02 (29)	2.6 ± 0.22 (29)*	0.02	NA	

Difference in family size between infected and uninfected lines was tested using Mann–Whitney two-sided test (***$P < 0.001$, *$P < 0.05$; all differences aside those in *Melittobia* remain significant after a Bonferroni correction for multiple comparisons). For virgin females, male mortality was estimated from 1 − (mean number of sons from virgin infected females/mean number of sons from virgin uninfected females).

Evidence of *Arsenophonus nasoniae* transfer between members of the filth fly guild in natural populations

Survey of *Arsenophonus* infection in members of the filth fly guild ($n = 403$ individuals, $n = 9$ species) through a PCR assay based on the *16S rRNA* gene revealed *Arsenophonus* infection in the previously described host *N. vitripennis*, and two novel host species: *Muscidifurax raptor* (Pteromalidae) and *Spalangia cameroni* (Pteromalidae), a species more distantly related to the two previous ones (Table S1). This observation demonstrates that the infection is widespread within the parasitoid community (three of nine species tested), although at low prevalence within infected species (1.2–8%). The prevalence ranges observed are in broad agreement with those previously estimated in western North American populations of *N. vitripennis* and *N. longicornis* (Balas *et al.* 1996). We also recorded the presence of *A. nasoniae* in five of 146 individuals of the birdnest fly *Protocalliphora azurea* (Calliphoridae), one of the usual host species of *Nasonia* spp.

We then used DNA sequence to investigate the phylogenetic relatedness of the *Arsenophonus* strains found in the different host species. *Arsenophonus* strains have multiply repeated *rRNA* gene copies in their genomes, preventing the use of *16S rRNA* gene sequences to discriminate closely related strains (Sorfova *et al.* 2008; Novakova *et al.* 2009). We therefore developed a multi locus approach, examining the sequence of three orthologous genes, *fbaA*, *ftsK* and *yaeT*, present in single copy in the *Arsenophonus* genome. We applied this typing to the *Arsenophonus* isolates discovered in this study, and to ten other *Arsenophonus* isolates from various host origins. The sequences obtained from *Arsenophonus* from the filth fly parasite species *M. raptor* and *S. cameroni* and from the birdnest fly *P. azurea* proved to be strictly identical over 1577 bp to those of the son-killer *A. nasoniae* strain isolated from *N. vitripennis*. Phylogenetically, these strains form a recent group confined to filth fly parasites, with only one other strain falling in this clade: an *Arsenophonus* strain from the pteromalid parasite *Pachycrepoideus vindemmiae* (Pteromalidae), a very polyphagous parasitoid that is a facultative parasite of filth fly pupae (Fig. 1). The very close relatedness of these strains rules out co-speciation with the host. Rather, the data imply a very recent (and potentially ongoing) mobility of *A. nasoniae* within the filth fly parasitoid community, including the filth fly host itself. We can also rule out horizontal transmission by introgression following hybridization. Introgression would transfer both bacterium and its associated mitotype from species to species (Hurst & Jiggins 2005). We rejected this suggestion because sequences of both COI mitochondrial and *18S rRNA* nuclear genes were strictly identical between infected and uninfected individuals within each parasitoid species, but distinct between species. This leaves infectious transmission between species the most parsimonious explanation for the occurrence of very similar strains in distinct (and in some cases quite phylogenetically disparate) host species.

DISCUSSION

Whilst the ultimate origin of all symbionts is from a free living ancestor, lateral transfer of an existing symbiont from one species into another represents the most common

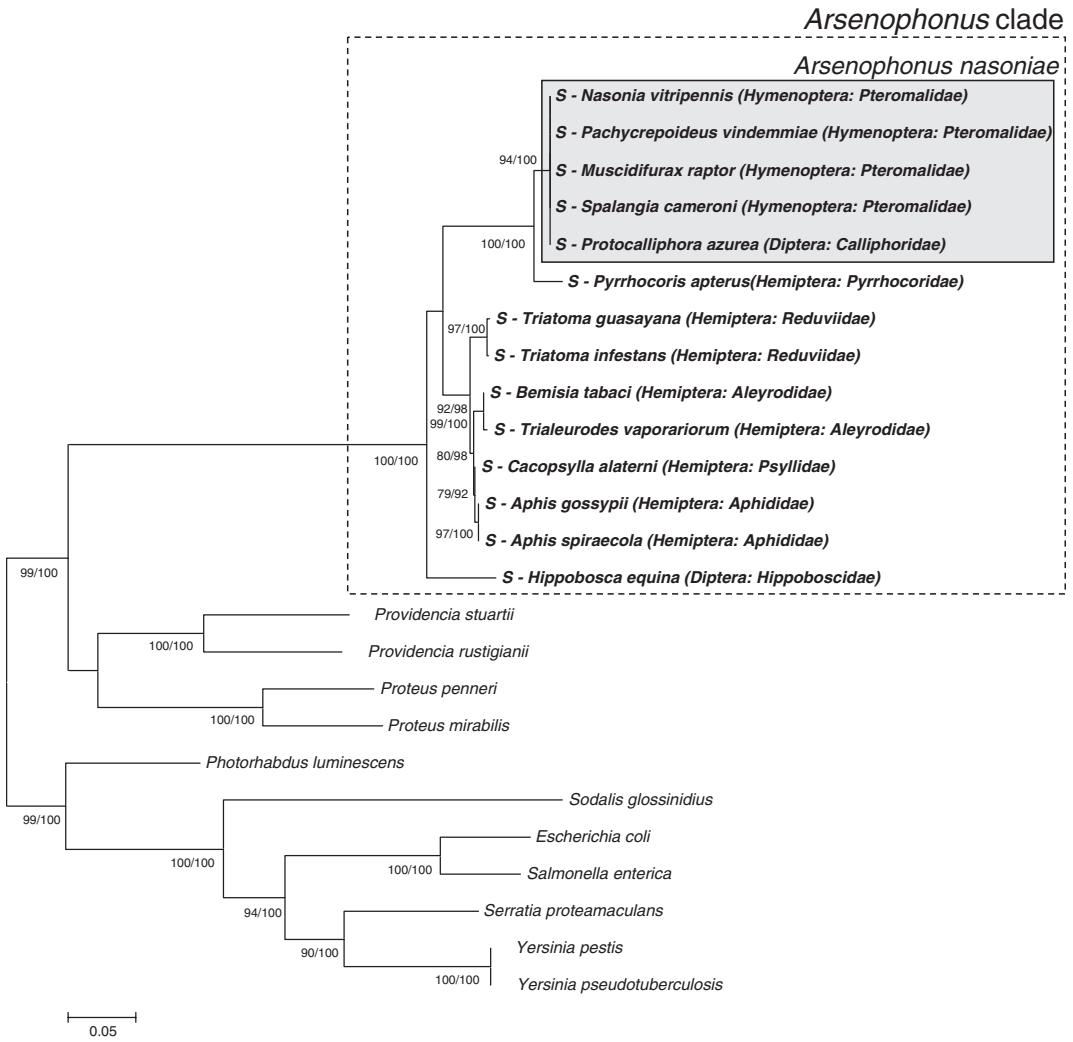

Figure 1 *Arsenophonus* phylogeny constructed using maximum likelihood (ML) estimations based on concatenated sequences of the *fbaA*, *ftsK* and *yaeT* genes. Host species of *Arsenophonus* are reported with *S* prefix. Eleven other major members of Enterobacteriaceae family were included in the analysis to delineate the *Arsenophonus* clade: *Escherichia coli* (CP000970), *Photorhabdus luminescens* (BX571864), *Proteus mirabilis* (AM942759), *Proteus penneri* (ABVP01000000), *Providencia rustigianii* (ABXV01000000), *Providencia stuartii* (ABJD02000000), *Salmonella enterica* (CAAT01000000), *Serratia proteamaculans* (CP000826), *Sodalis glossinidius* (AP008232), *Yersinia pestis* (CP000668) and *Yersinia pseudotuberculosis* (CP000950). Bootstrap values higher than 70% are shown at the nodes. For nodes also supported by Bayesian inferences (BI), the corresponding posterior probability is shown after the bootstrap value obtained by ML estimations.

process by which new symbioses are established. However, lateral transfer has previously been considered important only in the initial establishment of symbioses, and as a determinant of the frequency of infection in a community (Engelstadter & Hurst 2006). This is because previous studies have indicated that either the contacts themselves (e.g. blood–blood contact in woodlice: Rigaud & Juchault 1995), or the rate of transfer following contact (few exposure events producing stable infections, e.g. *Spiroplasma* transfer between *Drosophila* through mites, *Wobachia* transmission from parasitoid to host or between parasitoids: Heath *et al.* 1999; Huigens *et al.* 2000, 2004; Jaenike *et al.* 2007) are sufficiently rare that the short term ecological and evolutionary dynamics of these systems approximated to a single host/single symbiont framework based on pure vertical transmission. In our study, we have demonstrated that for *A. nasoniae*, a commonly occurring exposure known to produce intraspecific transmission results in very efficient symbiont transfer between species. Our data collectively represent the first case study, where experimental transfer of a selfish genetic trait between members of an ecological community occurs freely on contact, through a mechanism

of transmission that is known to occur commonly in the community, to produce stable vertically transmitted infections in the new species, with evidence that the process has occurred in a natural community. The principle conclusion from this is that it cannot be assumed that a single host species/single symbiont framework is satisfactory; rather, the assumption of low lateral transfer requires validation.

An examination of the likely rate of the horizontal transfer in the *A. nasoniae*/wasp system is instructive in assessing the problems with neglecting this process in our understanding of its population and evolutionary ecology. Recent field estimates from bird's nest populations of *Nasonia* in the Netherlands and Germany indicate 40% of pupae carry *N. vitripennis* from more than one *N. vitripennis* matriline (Grillenberger *et al.* 2008). In Eastern North America, where *N. vitripennis* co-occurs in bird's nests with *N. giraulti*, 29% of nests were colonized by both *N. vitripennis* and *N. giraulti* (with the remainder colonized by *N. vitripennis* alone) (Grillenberger *et al.* 2009). Study of a subsample of nests, where both wasps were present indicated 42% of pupae were parasitized by more than one wasp species, which accords with laboratory experiments indicating that aversion to multiparasitism is not strong (Ivens *et al.* 2009). For an *N. vitripennis* female, the rate of multiparasitism can be estimated as the product of nests inhabited by both species and rates of multiparasitism within those nests. This produces an estimated multiparasitism rate of 12%. For an *N. giraulti* individual, the rate of multiparasitism in nature is nearer 40%, as *N. giraulti* individuals were rarely found as the sole species in a nest, and thus always subject to multiparasitism pressure (Grillenberger *et al.* 2009). In other settings, where filth flies are common, such as agricultural animal facilities, field study estimated that between 2 and 12% of pupae contain more than one species of wasp, with a wider diversity of wasps being found in multiparasitism (Klunker 1994; De Carvalho *et al.* 2005).

These rates of multiparasitism, combined with the efficiency of transfer between matrilines demonstrated in this study, are clearly sufficient to shuffle symbiont strains between species each and every generation, and require the coevolutionary interaction to be considered as one son-killer interacting with a variety of host species. The rate of interspecific transfer is also high enough to warrant incorporation into population biology models of the system. It is certainly the case that new infections in *N. giraulti* will commonly arise associated with sharing pupal host with *N. vitripennis*. Indeed, the rate of lateral transfer is sufficient that even should infections lack 'drive' in the new host species, the steady 'drip' through lateral transfer may be sufficient to maintain infection at low prevalence in the 'new' host species.

An interesting additional possibility is that horizontal transfer may be sufficiently common to regularly generate coinfection of *A. nasoniae* within an individual wasp, both from intra and interhost species lateral transfer. The rate at which coinfection occurs will depend on whether the rate of lateral transfer is affected by the presence of a resident strain, a parameter that requires experimental evaluation. Providing this is possible, the frequency of coinfection in wasps would depend additionally on the rate at which multiple infections segregate. Coinfections are potentially important as avenues through which genetic material can exchange between symbiont strains, for instance through phage transfer (Darby *et al.* 2010). Where infectious transmission occurs, coinfection can also be accompanied by selection for increased virulence.

It is our conjecture that *A. nasoniae* likely represents a global partner of chalcid wasps, commonly exchanging within communities through multiparasitism, and occasionally exchanging between communities through 'bridge' species which occasionally take hosts from outside their community. This is supported by the observation of infection in *P. vindemmiae*, an ectoparasite species from outside the filth fly guild. However, we also expect *A. nasoniae* presence to be restricted by dint of its vertical transmission mechanism (Huger *et al.* 1985; Skinner 1985), to idiobiont parasitoid wasps (wasps that kill their hosts at oviposition), and occasionally the wasps' hosts (Duron *et al.* 2008).

It is interesting to speculate as to why *A. nasoniae* shows this pattern of common and easy lateral transfer and other bacteria do not. Most bacteria that transmit vertically, such as *Wolbachia*, have lost the ability to grow outside of the host following long periods of coevolution with arthropods (e.g. Dobson *et al.* 2002). *Arsenophonus nasoniae* is distinct in being able to grow in cell free media, albeit cell free media with supplements (Werren *et al.* 1986). Parallel to this, its genome suggests a relatively broad range of metabolic functions, as well as the ability to invade through epithelia and manipulate host immune systems (Darby *et al.* 2010; Wilkes *et al.* 2010). It is likely that these capabilities are maintained because of the natural transmission cycle of the bacterium. It is placed in the fly host pupa on oviposition, replicates within it, and is then taken up perorally by wasp larvae (Huger *et al.* 1985). *Arsenophonus nasoniae* is adapted to both saprophytic replication in the fly host and invasion of the living wasp. Interspecific transmission is relatively straightforward for such a bacterium compared to those that are transovarially transmitted, such as *Wolbachia*, where processes of invasion into the host do not occur each generation and where the bacterium is exposed to a single, highly regulated environment. From considerations such as these we would predict that interspecific transfer will occur most readily for symbionts that either maintain a non-symbiotic phase in their life cycle, or which have a very recent free living ancestor. For these symbionts, direct

introduction of the bacterium into the hemocoel is not a prerequisite for lateral transfer, as the symbiont can still transit through epithelia.

The finding that lateral transfer occurs freely for this bacterium following exposure contrasts with the incidence of *A. nasoniae* amongst species. Despite having 'free' lateral transfer on a per contact basis, *A. nasoniae* is present in only a fraction of the species we surveyed, and was also absent in previous surveys of *N. giraulti* (Balas *et al.* 1996). Indeed, the number of species in the clade infected with *Wolbachia* is probably greater, gauging from the presence of *Wolbachia* in all species of *Nasonia* (Raychoudhury *et al.* 2009). Thus, *Wolbachia* appears at least as successful in colonizing the clade despite being much less likely to transfer on a per contact basis.

There are probably two resolutions to this 'paradox'. First, there may be many more exposure events to *Wolbachia*, which overcomes the low per contact transmission probability. In this regard, it is notable that, with the exception of the filth fly itself, *A. nasoniae* has not been found outside of parasitic wasps making the source pool for infection with *Wolbachia* (other arthropod hosts) much larger than that for *A. nasoniae* (chalcid wasps). The second feature that may limit *A. nasoniae* presence in natural communities is failure to invade following lateral transfer. Failure to spread may occur because the infections do not possess a relevant drive trait, or because infections incur a direct cost. It is notable that son-killing trait will in fact be neutral in some host species (for instance in those where females lay a single egg in a host) and can be disadvantageous to the bacterium in others (as it renders the host prone to being unmated, and thus not producing daughters). It is also notable that inherited symbionts seem to vary in their physiological impact on novel hosts. Whilst no direct costs to infection with *Wolbachia* have been observed following transinfection, *Spiroplasma* infections commonly have a very deleterious effect on novel hosts (Tinsley & Majerus 2007). Future work should investigate the impact of *A. nasoniae* on the fitness of novel hosts. In particular, it will be important to ascertain whether the spread of these infections is impeded by any negative fitness impacts that emerge in novel host species.

In conclusion, the results of a variety of studies have indicated how lateral transfer of symbionts between arthropods can be a potent source of novelty, both adaptive and selfish. In evolutionary biology, the traditional source of novelty has been mutation of genes within the nuclear genome. However, as a mutational source that differs both in frequency and complexity, the evolutionary biology of symbiont induced traits requires special treatment. Understanding the evolutionary biology of laterally transmitted symbionts is thus a pressing goal in developing our understanding of arthropod evolutionary ecology.

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AUTHOR CONTRIBUTIONS

Experiments were designed by OD, TW and GH. OD and TW performed the research. OD, TW and GH analysed data. OD, TW and GH wrote the paper.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Table S1 Distribution of *Arsenophonus* amongst parasitoid wasps and their host flies. Infection was evidenced through PCR amplification of a fragment of *Arsenophonus 16S rRNA* gene. One parasitoid female per host fly pupa has been checked for infection, minimizing the risk of sampling sibling individuals.

Table S2 Genes and primer features. For reference to the *Arsenophonus nasoniae* genome ORFs, see Darby *et al.* (2010).

Table S3 Amplifiability, diversity and characteristics of the three MLST genes from typing of 14 *Arsenophonus* isolates. Two genes (*ftsK* and *yaeT*) showed positive PCR amplification for all the *Arsenophonus* isolates, but one for 13 isolates (*fbaA* with the *Arsenophonus* strain of the fire bug *Pyrrhocoris apterus* failing to amplify). The DnaSP program (Librado & Rozas 2009) was used to calculate the number of polymorphic site, the %GC content, and the pairwise ratio of non-synonymous to synonymous substitutions (K_a/K_s). We used Sawyer's test procedure (Sawyer 1989) implemented in GENECONV (Sawyer 1999) to perform statistical analysis for intragenic recombination (10 000 permutations).

Table S4 Maternal inheritance of *Arsenophonus nasoniae* infection through generations F3–F6 post-transfer (F1 and F2 are presented in Table 1). Infection was evidenced through PCR amplification of a fragment of *A. nasoniae 16S*.

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