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Loss of microbial (pathogen) infections associated with recent invasions of the red imported fire ant *Solenopsis invicta*

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Abstract Loss of natural enemies during colonization is a prominent hypothesis explaining enhanced performance of invasive species in introduced areas. Numerous studies have tested this enemy release hypothesis in a wide range of taxa but few studies have focused on invasive ants. We conducted extensive surveys for the presence of six microbes in recently established populations (California, Australia, New Zealand, Taiwan, and China) of the invasive fire ant *Solenopsis invicta*. These microbes include *Wolbachia*, two microsporidia (*Kneallhazia solenopsae* and *Vairimorpha invictae*) and three RNA viruses (SINV-1, -2 and -3), all of which previously have been reported in native South American populations of *S. invicta*. These surveys showed that the total number of enemy species is lower in the recently invaded areas compared with both South American and US populations. Only two microbes were found in any of these recently invaded areas: SINV-1 was detected in all surveyed populations except Australia and New Zealand, and SINV-2 was detected in California and Taiwan only. These results support the general prediction that invasive species lose many of their natural enemies during invasion. Further, the conspicuous absence of some of these microbes in these areas may result from strong selection against founders due to fitness costs associated with harboring detrimental infections rather than the alternative hypothesis that they simply were absent among the original founders. While the successful invasion of *S. invicta* in these recently invaded areas may be explained partly by the absence of natural enemies, other factors likely have been important as well.

Keywords Enemy release hypothesis · Invasive species · Microsporidia · *Solenopsis invicta* · RNA virus · *Wolbachia*

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Introduction

Biological invasions pose major threats to local biodiversity, ecological functioning, public health, and the economy (Parker et al. 1999; Mack et al. 2000). In response to ever increasing numbers of

invasive species and a desire to mitigate these incursions, it is not surprising that research aimed at understanding the critical biotic and abiotic factors attributing to successful invasions has increased dramatically in the last two decades (Dobson 1988; Settle and Wilson 1990; Callaway and Aschehoug 2000; Torchin et al. 2001). One common explanation for success of many invasive species is the enemy release hypothesis (ERH) (Mitchell and Power 2003; Torchin et al. 2003; Torchin and Mitchell 2004). This hypothesis predicts that invasive species often leave behind many co-evolved natural enemy species found in their native ranges (e.g., predators, parasites, competitors, and pathogens), and the release from these natural enemies allows the invading species to actually perform better and reach higher densities in its new environment (Prenter et al. 2004). An extensive body of literature exists testing the ERH across animal and plant taxa, and these studies generally support the ERH showing a general trend of invasive species escaping 50–90% of their native enemies (Mitchell and Power 2003; Torchin et al. 2003; DeWalt et al. 2004; Marr et al. 2008; Adams et al. 2009; Cincotta et al. 2009; Ross et al. 2010).

Several studies also have suggested a possible role of the release from natural enemies in the success of invasive ant species (McGlynn 1999; Holway et al. 2002), which include some of the world's most damaging invasive pests. Among these, the fire ant *Solenopsis invicta* is arguably the most well studied invasive ant, and several lines of evidence are consistent with the ERH in explaining the success of this invasive ant in its introduced range in the US. Population densities of *S. invicta* in the US generally are five to seven times higher than densities in the species' native South American range, and these differences apparently are not due simply to abiotic factors such as climate conditions, soil types, or the frequency of the polygyne (multiple queens per nest) social form (Porter 1992; Porter et al. 1997). Further, survey data clearly have shown that natural enemy richness and abundance is considerably lower in the US compared with South America (Jouvenaz 1990; Porter et al. 1997).

On the other hand, several studies in this ant and other invasive ants suggest that factors other than release from natural enemies (e.g., greater competitive ability against native ants, changes in social behavior and colony genetic structure) also have been important for their invasive success (Porter and Savignano 1990; Holway et al. 1998; Tsutsui et al. 2000; Tsutsui and Suarez 2003; Yang et al. 2009). As one example, a comprehensive study by Cremer et al. (2008) led the authors to suggest that the invasive success of the garden ant *Lasius neglectus* can be attributed to the release from natural enemies (specifically, the endosymbiotic bacteria *Wolbachia* and the fungus *Beauveria bassiana*) as well as to a suite of pre-existing adaptations (e.g., colony social organization) that evolved in the species' native range. Interestingly, parallel patterns showing the near or complete absence of *Wolbachia* endosymbionts in introduced populations despite often high prevalence in native populations also have been reported for two other invasive ants, namely, the fire ant *S. invicta* and the Argentine ant *Linepithema humile* (Shoemaker et al. 2000, 2003; Holway et al. 2002; Tsutsui et al. 2003; Reuter et al. 2005). However, given all of these studies surveyed for at most two potential natural enemies (pathogens), broader surveys for additional microbes, particularly those thought or known to have negative impacts on host fitness, are needed to further evaluate the potential role of release from natural enemies in ant invasion success, especially compared with other potential factors that also undoubtedly play a role in invasion success. Indeed, despite the surveys for natural enemies attacking *S. invicta* described above, systematic surveys for microbes infecting this ant, especially in recently invaded areas (see below), are more limited or nonexistent.

For the current study, we conducted extensive surveys for the presence of six microbes (some of which are certainly detrimental to fire ants; Oi and Williams 2003; Valles et al. 2004a; Oi et al. 2005; Overton et al. 2006; Valles et al. 2007a; Valles and Hashimoto 2009) in areas where *S. invicta* has recently invaded in an attempt to fill this current gap in our knowledge. These six microbes include the endosymbiotic bacteria *Wolbachia*, two species of microsporidia (*Kneallhazia solenopsae* and *Vairimorpha invictae*), and three RNA viruses (SINV-1, SINV-2 and SINV-3). Our survey of the distribution and prevalence of these pathogens in newly invaded parts of the world, including in California, Australia, New Zealand, Taiwan and China (MacKay and Fagerlund, 1997; Buckley 1999; Nattrass and Vanderwoude 2001; Huang et al. 2004; Zhang et al. 2007), provides

the additional opportunity to test the generality of the ERH without the confounding effects of ongoing biological control projects (which obviously is the case for US *S. invicta*) (Porter et al. 2004; Vander Meer et al. 2007). In addition, a direct comparison of our survey results for *S. invicta* from the US and more recently invaded areas may potentially shed light on whether such infections can persist and spread during the early phases of establishment and spread of fire ants or whether such microbes are more likely to invade (possibly as a result of secondary host invasions) and persist only in rather large, relatively stable host ant populations, such as found in the southern US. Finally, our study represents the most comprehensive geographic survey for microbes in any single ant species to date (six microbes surveyed in 981 colonies from 13 collection sites in five introduced populations of *S. invicta*) and provides a framework for understanding the factors responsible for the success of *S. invicta* and for developing future biological control strategies of this invasive ant.

Materials and methods

Collection of samples and RNA/DNA extractions

Colonies of *S. invicta* were collected from recently invaded areas summarized as follows: (1) California: Orange County (California site 1–3) and Riverside County (California site 4), with both sites result of recent outbreaks, (2) Australia: Richlands-Waco, Brisbane (Australia site 1) and Gladstone (Australia site 2), which likely represent independent introductions, (3) Taiwan: Taoyuan (Taiwan site 1) and Chiayi (Taiwan site 2), which also likely result from separate introductions, (4) China: Hong Kong (China site 1), ShenZhen, Guangdong (China site 2), WuChuan, Guangdong (China site 3) and BeiLiu, Guangxi (China site 4), (5) New Zealand: three nests from Whirinaki area (New Zealand site 1), a site where fire ants were successfully eradicated in 2006 and have not been found elsewhere to date (Fig. 1). Because our sample size from New Zealand is too small (three nests, Fig. 1) for conducting meaningful statistical analyses (yet this is likely the true sample size since these were intercepted and subsequently eradicated), data from this area are excluded from following analyses. However, our survey data for New Zealand are still treated as accessory information since our goal was to examine the occurrence of pathogens in all current introduced areas of *S. invicta*. Further details regarding geographic locations of collection sites, year of first detection at each site and numbers of colonies collected per site are presented in Fig. 1. None of the populations sampled from these areas have been associated with any intentional field releases of microbial control agents. Thirty to fifty workers per nest were collected and preserved into 95% alcohol and subsequently stored at −80°C pending molecular analyses. DNA and RNA extractions were performed on pools of 10–15 individual ants from each nest using the Puregene DNA extraction kit (Gentra Systems Inc., USA) and TRIzol Reagent (Invitrogen, USA), respectively, following the manufacturers' suggested protocols (Shoemaker et al. 2000; Valles et al. 2004a, b, 2007a; Valles and Hashimoto 2009).

Surveys for *Wolbachia* and microsporidia

DNA from bulk extracted ants served as template for screening for the presence of *Wolbachia* and two microsporidia (*K. solenopsae* and *V. invictae*) using microbe-specific polymerase chain reaction (PCR) assays. We surveyed for the presence of *Wolbachia* using oligonucleotide primers *Wsp*81F and *Wsp*691R (Zhou et al. 1998; Shoemaker et al. 2000) following the methods described in Bouwma et al. (2006). We also included two primers that amplify a portion of the nuclear EF1α gene (EF1α-532F and EF1α-610R) to serve as internal controls for DNA quality (described in Shoemaker et al. 2000). PCR cycling profile and subsequent electrophoresis methods were identical to those described by Shoemaker et al. (2000, 2003). An individual nest was considered *Wolbachia*-infected only if both a *Wolbachia*-specific fragment (ca. 600 bp) and EF1α fragment (ca. 400 bp) were amplified from the bulk extracted DNA. We further screened our DNA extracts from each nest for *Wolbachia* using the long PCR protocol (primers *Wsp*-F and *Wsp*-R) developed by Jeyaprakash and Hoy (2000). These authors suggested that this PCR assay is much more sensitive and accurate in detecting *Wolbachia* infections. However, our survey results were identical for these two different assays.

We used a multiplex PCR assay to screen the bulk extracted DNA samples simultaneously for the two

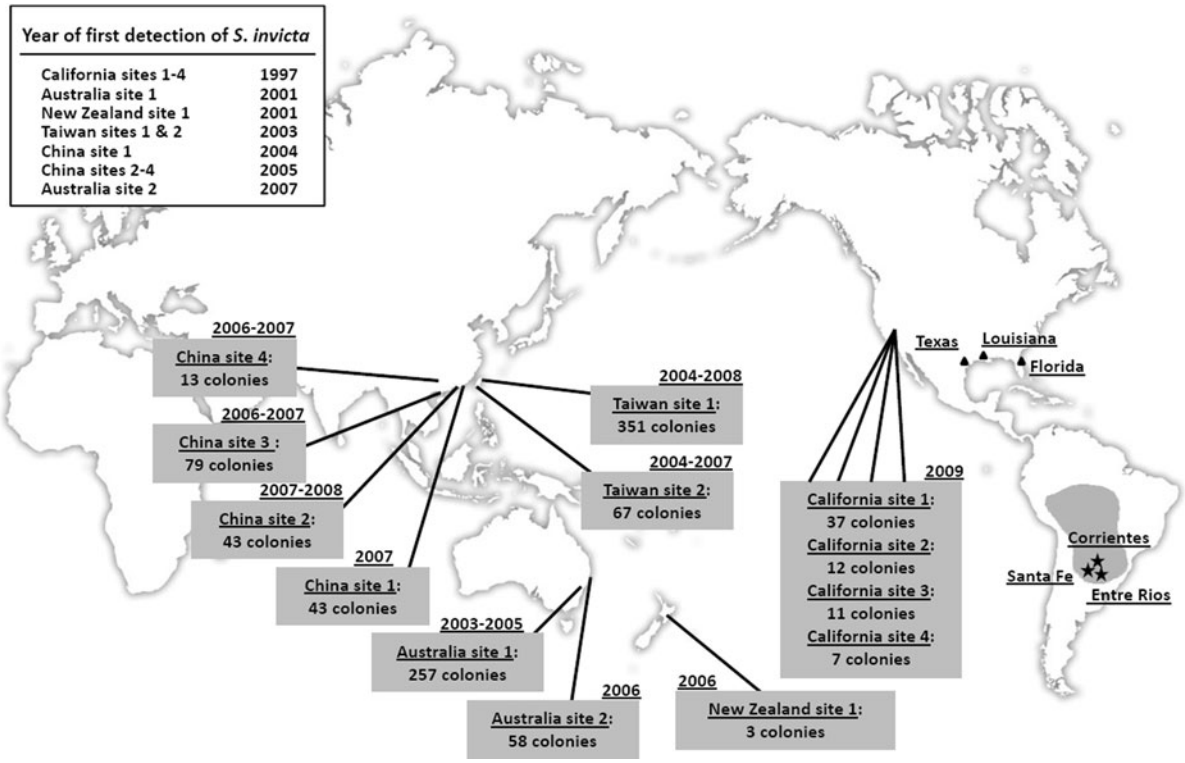

Fig. 1 Map showing the collection sites from recently invaded areas used in the present study. Number of colonies collected from each site is indicated in *gray boxes* and year of collection is shown above each box. *Triangles* and *stars* represent populations from the US and South America, respectively, where microbe richness and prevalence were estimated and used for comparative analyses in the present study. Native South American range of *Solenopsis invicta* is shown in gray shading. Approximate time (year) of first detection of *Solenopsis invicta* in every newly introduced area is listed in the *box* located upper-left

microsporidia *K. solenopsae* and *V. invictae* following the detailed protocol and procedures reported in Valles et al. (2004b). Positive (infected ants), negative (uninfected ants) and blank (no template) controls were included in all PCR reactions mentioned above.

Surveys for SINV-1, SINV-2 and SINV-3 RNA viruses

Two-step reverse-transcriptase PCR (RT-PCR) was employed for detection of fire ant viruses SINV-1, SINV-2 and SINV-3. First strand cDNA was synthesized from total RNA by using SuperScript III reverse transcriptase (Invitrogen, USA) with oligo $dT_{(18)}$ following the manufacturer's instructions. Subsequent PCRs were carried out separately with primer sets that are specific for each virus following previous methods (Valles and Strong 2005; Valles et al. 2007a; Valles and Hashimoto 2009). These primers include P341 and P343 for SINV-1 (and a second distinct virus genotypes, SINV-1A) (Valles and Strong 2005), P64 and P65 for SINV-2 (Valles et al. 2007a), and P705 and P707 for SINV-3 (Valles and Hashimoto 2009). PCR reaction conditions and thermal cycling profiles were the same as those reported in Valles and Strong (2005), Valles et al. (2007a), and Valles and Hashimoto (2009). We considered a nest as infected with a given virus when a visible amplicon of the correct molecular weight for that virus was observed (1.3 kb for P341/P343, 319 bp for P64/P65, 72 bp for P705/P707, respectively). We included positive controls (known virus-infected ants), negative controls (uninfected ants) and no template controls in each PCR batch.

Sequence verification of the presence of microbes

We sequenced each PCR amplicon to further verify the PCR products that were amplified from a given PCR assay actually represented the targeted pathogen species. The general methods can be found in Ahrens and Shoemaker (2005). Briefly, PCR amplicons were gel purified, ligated into PCR4-TOPO vector, and transformed into TOP10 competent *Escherichia coli* cells (Invitrogen, USA). We then picked colonies directly, performed a second round of PCR, purified resulting PCR products, and then used these purified products as a template for cycle sequencing reactions performed using the ABI PRISM BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Calif.). Sequencing reactions were purified using CleanSEQ magnetic beads (Agencourt Bioscience) and run on an ABI 3730 sequencer at the National Taiwan University Core Laboratory for Sequencing. At least four clones for each pathogen found within a given sample were selected for sequencing. We ran the blastx (nucleotide) program (Altschul et al. 1990) to compare our sequences to those available in the non-redundant nucleotide database available in GenBank. Because the PCR assay for SINV-1 virus using the primer set P341/P343 amplifies a portion of the capsid gene region that generally is subject to a high mutation rate (Shackelton et al. 2005), it is possible that a given nucleotide sequence for this gene may not closely resemble existing sequences in GenBank for this gene from this virus because of a large number of mutations. Previous studies have shown that nucleotide sequence of this gene region differs substantially between SINV-1 or SINV-1A (Valles and Strong 2005), yet most of the mutational differences observed were synonymous so that the translated sequences had relatively high (97%) amino acid identity. Thus, we also ran the tblastx program (Altschul et al. 1990) to compare the translated sequences of putative SINV-1 nucleotide sequences to those available in the non-redundant protein database available in GenBank.

Microbe prevalence and richness in native and US *S. invicta*

We compiled survey data from multiple previous studies to determine the prevalence and richness of these target pathogens in the US and South American *S. invicta* since no single study to date has performed comprehensive surveys of these pathogens in these geographic areas. Two potential concerns or sources of bias with these sorts of analyses are that (1) different sampling strategies across different studies may bias the outcome of overall estimates (Colautti et al. 2004; Torchin and Mitchell 2004) and (2) lack of knowledge of the presumed source population means one cannot infer the proper native reference population for comparison (Colautti et al. 2004, 2005). This latter issue is important because there is likely to be considerable variation in enemy diversity and prevalence among native areas (host subpopulations likely harbor only a subset of the enemies found across the entire native range), so that confidently inferring whether natural enemy diversity is lower in an introduced area requires that one makes the proper comparison with the putative source population rather than the entire native range at large. We made several attempts to avoid these sources of bias. First, we included data only from studies that employed a sampling scheme of similar geographic area. Second, previous work has identified the most likely source population of US *S. invicta* as being at or near northern Argentina (Caldera et al. 2008). Based on these previous results, we excluded data from survey studies conducted in Brazil (a broad area that clearly appears not to be a potential source for any worldwide populations) and instead limited our analyses to survey data only from three nearby sites including Corrientes, Entre Rios and Santa Fe, Argentina (Fig. 1). Finally, while several lines of evidence suggest that other introduced populations likely come from the large US population (Henshaw et al 2005; Tschinkel 2006; Yang et al. 2008), some doubts remain (Yang et al. 2009; Ascunce et al., unpublished data), and it is still unknown which particular geographic area of the US might be the source population for these secondary introductions. Without such knowledge, we included all survey data from Florida, Louisiana and Texas since any are potential sources for these other areas and these are the only populations where all studied pathogen species have been surveyed and documented in the existing literature.

We used a non-parametric Kruskal–Wallis chi-square approximation to test for differences in enemy richness (number of enemies) and enemy prevalence (% of colonies infected) among *S. invicta* populations

from South America, the US and recent introduced areas (significance determined by non-overlap of 95% confidence intervals).

Results and discussion

We conducted an extensive survey of richness and prevalence of six microbes (pathogens) infecting the fire ant *S. invicta* collected from recently invaded populations including California, Australia, New Zealand, Taiwan, and China. Results of microbe-specific PCRs or RT-PCRs for the newly invaded populations revealed the absence of all microbes in these areas with two exceptions: SINV-1 was detected in sites from California, Taiwan and China and SINV-2 was detected in sites from California and Taiwan (Table 1). None of the pathogens were detected in either Australia or New Zealand. Analyses of our sequence data completely supported our survey data: the sequences for putative SINV-2 virus were very similar (98% identical) to previously published sequences for SINV-2 (GenBank accession number EF428566), whereas the sequences for SINV-1 from infected colonies were 83 and 84% identical to previously published SINV-1 and SINV-1A sequence, respectively (GenBank accession numbers AY634314, AY831776 and FJ229495). Further, a comparison of amino acid sequences revealed that these SINV-1-like sequences exhibited 93–95% identities with SINV-1 and SINV-1A. Therefore, these combined data suggest that all positive PCRs detected from our virus surveys using RT-PCR were robust and specific for SINV-1 and SINV-2, despite considerable geographic variation (data not shown). In summary, no microbes other than SINV-1 and SINV-2 were found in these recently invaded areas, and *S. invicta* from Taiwan and California are the only populations shown to harbor both viruses (Table 1).

Data on enemy richness and prevalence from previous studies conducted in the native range in Corrientes, Entre Rios and Santa Fe, Argentina and in the US (Florida, Louisiana and Texas) are shown in Table 2. A comparison of these data with our data indicates that fire ants in the more recently invaded areas harbor significantly fewer numbers of different microbes (1 ± 1.0) compared with either the US (3.67 ± 0.58) or native population (5.33 ± 0.58) (Kruskal–Wallis $\chi^2 = 8.17$, $df = 2$, $N = 10$, $P = 0.0169$). Further, microbe richness in the US is intermediate between that found in South America and recently introduced areas, and is significantly different from both (Wilcoxon Signed Rank test: $P = 0.036$ and 0.016, respectively). No significant difference is observed in the prevalence of SINV-1 and SINV-2 among the native and all introduced populations (Kruskal–Wallis $\chi^2 = 1.07$, $df = 2$, $N = 10$, $P = 0.5849$ for SINV-1; $\chi^2 = 0.35$, $df = 2$, $N = 10$, $P = 0.8378$ for SINV-2). We did not perform significant tests for prevalence of those pathogens that were absent in newly introduced areas to avoid redundancy (absence of a given pathogen implies zero prevalence).

The general patterns above indicate that recent invasions of *S. invicta* have been accompanied by a paucity of pathogen infections. Below we discuss potential mechanisms accounting for the conspicuous absence of some but not other microbes in these recently established populations as well as possible connections between successful invasion of *S. invicta* into these areas and loss of natural enemies (Porter 1992; Porter et al. 1997).

Table 1 Prevalence (mean % of surveyed colonies infected ± SD) of six microbes within recently established *S. invicta* populations

Species	California ($N = 4$, $n = 67$)	Australia ($N = 2$, $n = 315$)	Taiwan ($N = 2$, $n = 418$)	China ($N = 4$, $n = 178$)	New Zealand ($N = 1$, $n = 3$)
Wolbachia	0	0	0	0	0
Kneallhazia solenopsae	0	0	0	0	0
Vairimorpha invictae	0	0	0	0	0
SINV-1	19.2 ± 4.6	0	46.0 ± 11.3	41.0 ± 28.0	0
SINV-2	13.3 ± 4.1	0	16.7 ± 4.7	0	0
SINV-3	0	0	0	0	0

N number of collection sites, *n* total number of colonies collected from all sites in a given areas

Table 2 Prevalence (% of colonies infected) data for six microbes infecting *S. invicta*

Species	Native range	Prevalence (%)	Reference	USA	Prevalence (%)	Reference
Wolbachia	Corrientes	56.0–86.0	Shoemaker et al. (2000)	Florida	0	Bouwma et al. (2006)
	Entre Rios	24.0–71.0	Shoemaker (unpublished data)	Louisiana	0	Bouwma et al. (2006)
	Santa Fe	6.0–12.5	Shoemaker (unpublished data)	Texas	0	Bouwma et al. (2006)
Kneallhazia solenopsae	Corrientes	28.0	Briano et al. (2006)	Florida	0–95.5	Oi et al. (2004)
	Entre Rios	3.0	Briano et al. (2006)	Louisiana	2.8–71.4	Milks et al. (2008)
	Santa Fe	6.9	Briano (2005)	Texas	9.0–47.0	Cook (2002)
Vairimorpha invictae	Corrientes	7.0–7.7	Briano et al. (2006)	Florida	0	Oi and Valles (unpublished data)
	Entre Rios	7.0	Briano et al. (2006)	Louisiana	0	Oi and Valles (unpublished data)
	Santa Fe	9.9	Briano et al. (2006)	Texas	0	Oi and Valles (unpublished data)
SINV-1	Corrientes	25.0	Valles et al. (2009)	Florida	10.0–52.0	Valles et al. (2007b)
	Entre Rios	13.3	Valles et al. (2009)	Louisiana	13.3–20.0	Valles et al. (2007b, 2009)
	Santa Fe	6.9–28.9	Valles et al. (2009)	Texas	4.8–67.0	Valles et al. (2007b, 2009)
SINV-2	Corrientes	16.6	Valles et al. (2009)	Florida	1.6–16.4	Hashimoto and Valles (2008)
	Entre Rios	6.7	Valles et al. (2009)	Louisiana	0	Valles et al. (2009)
	Santa Fe	3.4–15.8	Valles et al. (2009)	Texas	25.4	Valles et al. (2009)
SINV-3	Corrientes	0	Valles et al. (2009)	Florida	15.0	Valles (unpublished data)
	Entre Rios	0	Valles et al. (2009)	Louisiana	20.0	Valles et al. (2009)
	Santa Fe	1.7–7.9	Valles et al. (2009)	Texas	17.5	Valles et al. (2009)

Data are from previous studies conducted in South America and the US. Detailed collection information (geographic scale and sample size) can be found in original studies listed above (see references)

Loss of microbes (and other natural enemies) during invasion

The invasion of new species may serve as a filter for reducing the number of natural enemies in recently invaded areas, as we show for microbes infecting *S. invicta* in the present study. Two prevailing mechanisms to explain the lack of pathogens (and other natural enemies) are that they simply are absent among the original invading host individuals, presumably as a result of chance due to sampling a fewer number of host individuals from the source population, or that they are present initially in some proportion of the original founders but are lost due to drift or selection against infected individuals as a result of a fitness cost associated with harboring pathogens (Torchin et al. 2002; Torchin and Mitchell 2004).

The first mechanism is plausible in the case of *S. invicta* given the fact that previous population genetic studies suggest that relatively few numbers of founder queens likely were associated with introductions into these areas (Henshaw et al. 2005; Yang et al. 2008). This scenario is obviously the most parsimonious explanation for the absence of *Wolbachia* and *V. invictae* in these areas since the prevalence of these two microbes is quite low (perhaps even zero in the case of *V. invictae*, Oi and Valles, unpublished data) throughout the US, which appears to be most likely the source for these recent invasions (Shoemaker et al. 2000, 2003; Bouwma et al. 2006; Oi and Valles 2008). Thus, the absence of these two microbes, and perhaps others, in these areas may simply result from chance due to the sampling of relatively few numbers of individuals to serve as new founders from a source

population where most individuals lack these infections.

However, in the case of the RNA viruses and *K. solenopsae*, the alternative possibility that these microbes are absent in some or all recently established populations even though some proportion of the original founders were infected cannot be excluded. In this case, the absence of infections may stem from subsequent loss after invasion due to drift or, more likely, direct selection as a result of harboring a detrimental microbe. The presence of SINV-1 and SINV-2 in some of these populations but the complete absence of SINV-3 virus and the microsporidia *K. solenopsae* are consistent with differences in the direct fitness effects of these microbes on their hosts. How fitness effects shape the distribution of these microbes worldwide is further discussed below.

Both SINV-1 and SINV-2 viruses are found at intermediate to high prevalence in Taiwan and California, as well as in China in the case of SINV-1. Mitchell and Power (2003) found that invasive plants were more likely to be free of fungi compared with viruses because some viruses are less virulent and can even be systemic and persist with unapparent symptoms unlike the fungal pathogens. Consistent with this notion, recent studies have found both SINV-1 and SINV-2 infections are often asymptomatic over time, and for reasons poorly understood only occasionally lead to high brood mortality in infected colonies (Valles et al. 2004a, 2007a). Also, the additional findings that SINV-1 and SINV-2 infections may occur in all the castes and developmental stages (Hashimoto and Valles 2007, 2008), and are widely distributed and reach high levels of prevalence across the US (Valles et al. 2007b, 2009), further suggest these viruses are only occasionally detrimental to their hosts and, thus, are capable of hitch-hiking with its host species during an introduction as relatively neutral passengers (Mitchell and Power 2003).

In contrast, previous studies have demonstrated that both the SINV-3 virus and the microsporidia *K. solenopsae* have more consistent negative impacts on fire ant colony fitness (Williams et al. 1999; Oi and Williams 2002, 2003; Cook et al. 2003; Oi et al. 2005; Overton et al. 2006; Valles and Hashimoto 2009; Valles et al. 2009). While the extent of effects of these two microbes on the establishment of fire ants during early stages of invasion are unexplored, both empirical studies and theoretical simulations suggest there is a much lower probability of maintaining detrimental pathogens in small host populations (reviewed in Lloyd-Smith et al. 2005), so that the risk of pathogen loss in the early stages of host spread and establishment is high. Valles and Hashimoto (2009) have shown that SINV-3 infections are much more virulent than both SINV-1 and SINV-2 infections, and consistently result in high host mortality. The alternative suggestion that the absence of SINV-3 in these recently invaded areas stems from its absence among the original founders is further weakened by the fact that the prevalence of this virus in the US is similar to that of the other two viruses (Valles et al. 2009). Therefore, we consider a more likely explanation for its absence in these areas is that its higher pathogenicity has led to the elimination of this pathogen in these areas as a result of strong selection against SINV-3-infected founders (nonetheless, we acknowledge some caution is warranted given more extensive survey data suggesting that SINV-3 is more patchily distributed and has a relatively lower inter-colony infection rate compared with the other two viruses). Future empirical studies coupled with epidemiological models may shed light on this issue, especially given that this virus is found in the introduced US populations of *S. invicta*.

Along these lines, one point of consideration regarding the presence of SINV-3 (and *K. solenopsae*) infections in the US but its apparent absence in more recently invaded areas is that while these infections (and many others) may not be able to persist and spread during the early phases of establishment and spread of fire ants they clearly can invade and persist in rather large, relatively stable host ant populations (such as found in the southern US), perhaps arriving as a result of secondary host invasions in which some proportion of individuals harbor one or more such infections. Indeed, while five of the six pathogens surveyed are found within US *S. invicta*, it remains unclear which of these microbes, if any, were present among some proportion of the original founders and which microbes instead arrived much later as a result of secondary invasions of *S. invicta*. Several lines of evidence are consistent with additional inadvertent introductions of *S. invicta* into the US since its initial landfall in the 1930 s (Vinson 1997; Shoemaker et al. 2006; Caldera et al. 2008) and the same holds for some of the associated microbes. For example, the most

parsimonious explanation for the very low prevalence of *Wolbachia* infections within a single US population near a major point of entry (Pascagoula, Mississippi) is that these infections are the result of a secondary introduction of *S. invicta* into the US in which some proportion of individuals harbored these infections (see Bouwma et al. 2006 for additional evidence for this hypothesis). The late discovery and sporadic distribution of *K. solenopsae* may be for similar reasons and this scenario may well apply to the RNA viruses, especially SINV-3. This also may explain the absence of the putatively more detrimental microbes (e.g., the absence of SINV-3) in recently invaded areas (within the last decade or so) where the population densities of *S. invicta* are lower and the ant is still in the early stages of spreading. Such a scenario also would mean that as host population densities increase over time in these areas, it may only be a matter of time before these infections become established as a result of ongoing propagule pressure.

Release from natural enemies and invasion success of fire ants

Our data suggest recently established *S. invicta* populations have experienced decreased pressure from natural enemy species (pathogens) with strong fitness effects compared with those that are less detrimental. As the ERH predicts, invasive species likely perform better in the absence of virulent pathogens in introduced areas. However, the degree to which this release from pathogens affects invasion success of fire ants is unknown or impractical, especially given the large scale efforts to eradicate fire ants in these areas (McCubbin and Weiner 2002; Huang et al. 2004; Zhang et al. 2007). In spite of this shortcoming that cannot be overcome, the data here still provide convincing evidence that lack of pathogens frequently is associated with recent introductions of *S. invicta*. Such release from pathogens likely contributes to the invasion success of this ant, but other associated factors including human disturbance (King and Tschinkel 2008), superior competitive ability against local ants (Porter and Savignano 1990), and social form composition in the original founder population (Yang et al. 2009) are also likely to be important.

From an applied perspective, the importation and establishment of detrimental pathogens that currently are absent in recently introduced areas (e.g., SINV-3. *V. invictae*, and *K. solenopsae*) represents one potentially effective biological control management strategy for fire ants (as further evidenced by the theoretical foundation of ERH and past successful cases for other invasive organisms; reviewed in Keane and Crawley 2002). Such introductions of more pathogens potentially could have both direct effects on their hosts as well as enhanced effects resulting from the synergism of simultaneous pathogen infections. Observations of *S. invicta* colonies with dual infections of *K. solenopsae* and *V. invictae* have indicated potentially faster colony declines (Williams et al. 2003; Briano 2005). Furthermore, while some of the recently established populations of *S. invicta* harbor SINV-1 and SINV-2 viruses (only SINV-1 in China), which typically persist in the host as unapparent infections, both viruses can become virulent under certain stressful circumstances such as the presence of additional pathogens (Valles et al. 2004a, b, 2007a, b; Oldstone 2006). Introduction of SINV-3, *K. solenopsae,* or other pathogens in these areas therefore may have additional negative indirect fitness impacts on fire ants and facilitate declines in *S. invicta* population sizes.

Finally, the presence of SINV-1 and SINV-2 viruses in some worldwide populations also provides the opportunity to study sequence variation of the viruses in different populations in an attempt to reconstruct the global invasion history of this ant. Indeed, considering these introductions in many areas are recent events (less than two decades), the especially high mutation rate of SINV-1 (a typical feature of positive-strand RNA viruses) may provide finer resolution of host genetic structure and introduction history compared with traditional data generated from microsatellite markers and mtDNA sequences (Biek et al. 2006; Oi and Valles 2008). Preliminary sequence data from our laboratory reveal substantial divergence and geographical variation exists among SINV-1 viral sequences from host ants collected from South America, the US, and recent introduced areas, supporting the potential utility of analyzing sequence variation of this virus for this important task (Yang et al., unpublished data).

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