The host-pathogen interaction and its pest management implication: dicistroviruses and invasive ants as a model

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1.1 Invasive ants

Worldwide transfer of invasive ants has caused significant economic losses and ecological disturbances as their populations quickly established and usually displaced local ants as well as caused impacts on other arthropods (Holway et al., 2002; Human and Gordon, 1996; Touyama et al., 2003). To date, five ant species, such as red imported fire ant (*Solenopsis invicta*) and yellow crazy ant (*Anoplolepis gracilipes*), had been documented in the list of "100 of the world's worst invasive alien species" (Lowe et al., 2000). These invasive ants share few features which may facilitate their invasion success. For instance, these ant species construct a large colony with multiple queens, and their dispersal replies on colony budding (Abbott, 2006; Beardsley et al., 1979; Macom and Porter, 1996). These features also pose challenges to efficient management of invasive ants.

1.2 Invasive ant managements

Given the international trade and transport has been a major pathway for biological invasion (Yang et al., 2012), successful eradication of invasive ants is largely dependent on monitoring and eliminating nest at the early stage of establishment (Wylie et al., 2020; Yang et al., 2012). Except for the five invasive ant species by the IUCN (Lowe et al., 2000), Fournier et al. (2019) illustrated additional eight ant species that share similar features with current invasive ants (Fournier et al., 2019). These results imply that a full-fledged surveillance system at invasion hotspots, such as port and cargo area, is needed to reduce the invasion success of these invasive ants. In addition, once established, chemical treatments, such as nest injection and bait broadcasting, are commonly used for controlling invasive ant. An effective approach for controlling fire

ant, for example, is to use a two-step method, which alternates between bait broadcasting (a bait product usually contain insect growth regulators or actual toxins) and individual mound treatment (Riggs et al., 2002). Owing to the costs of chemical treatments and negative impacts on native ants, the reduction of insecticide use through integrated biological control likely mitigates the ecological damage of these invasive ants and also raises control efficiency. Recent efforts had been made to discover the biological agents of invasive ants for an integrated ant management frameworks, including parasitoids, bacteria, and viruses (Cooling et al., 2017; Drees et al., 2012; Lester et al., 2019; Woolfolk et al., 2016).

1.3 Dicistroviruses found in invasive ants

Due to the serial invasion history, the symbiont communities of invasive ants may be lost during invasion and/or gained with increasing time since invasion (Lester et al., 2017; Yang et al., 2010). The viral family *Dicistroviridae* is considered as one of the dominant viral families found in ants (Cooling et al., 2017; Johansson et al., 2013; Sébastien et al., 2015; Valles et al., 2004). The dicistrovirus has a positive-sense, single-stranded RNA genome encoding two non-overlapping open reading frames, and the genome ranges from 8 to 10 kb in size (Bonning and Miller, 2010). Most of dicistroviruses represent as chronic infection in the gut epithelium of host, and viral particles can be transmitted through food exchange (trophallaxis) between individuals as well as infected queen to offspring (Chen et al., 2006; Hashimoto and Valles, 2007). Given that the gut epithelium and gut microbiota of host play an important task of preventing penetration of pathogens (Jakubowska et al., 2013), the host-pathogen interaction is likely to determine the outcomes of infections, such as host immune response to pathogenic infections, and even infection-related mortality (Lester et al., 2019; Valles et al., 2004; Wang et al., 2018). In this thesis, I aim to characterize host-

pathogen interactions using the two invasive ants (red imported fire ant, *Solenopsis invicta* and yellow crazy ant, *Anoploplepis gracilipes*) address why infection of dicistroviruses is generally widespread in the introduced populations.

1.4 References

Abbott, K.L., 2006. Spatial dynamics of supercolonies of the invasive yellow crazy ant, *Anoplolepis gracilipes*, on Christmas Island, Indian Ocean. Divers. Distrib. 12, 101-110.

Beardsley, J.W., Jr., Su, T.H., McEwen, F.L., Gerling, D., 1979. Field investigations on the interrelationships of the big-headed ant, the gray pineapple mealybug, and pineapple mealybug wilt disease in Hawaii. Proc. Hawaii. Entomol. Soc. 24, 51-67.

Bonning, B.C., Miller, W.A., 2010. Dicistroviruses. Annu. Rev. Entomol. 55, 129-150.

Chen, Y., Evans, J., Feldlaufer, M., 2006. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. J. Invertebr. Pathol. 92, 152-159.

Cooling, M., Gruber, M.A.M., Hoffmann, B.D., Sebastien, A., Lester, P.J., 2017. A metatranscriptomic survey of the invasive yellow crazy ant, *Anoplolepis gracilipes*, identifies several potential viral and bacterial pathogens and mutualists. Insect Soc. 64, 197-207.

Drees, B.M., Calixto, A.A., Nester, P.R., 2012. Integrated pest management concepts for red imported fire ants *Solenopsis invicta* (Hymenoptera: Formicidae). Insect Sci. 20, 429-438.

Fournier, A., Penone, C., Pennino, M.G., Courchamp, F., 2019. Predicting future invaders and future invasions. Proc. Natl. Acad. Sci. U. S. A. 116, 7905-7910.

Hashimoto, Y., Valles, S.M., 2007. *Solenopsis invicta* virus-1 tissue tropism and intra-colony infection rate in the red imported fire ant: a quantitative PCR-based study. J. Invertebr. Pathol. 96, 156-161.

Holway, D.A., Lach, L., Suarez, A.V., Tsutsui, N.D., Case, T.J., 2002. The causes and consequences of ant invasions. Annu. Rev. Ecol. Evol. Syst. 33, 181-233.

Human, K.G., Gordon, D.M., 1996. Exploitation and interference competition between the invasive Argentine ant, *Linepithema humile*, and native ant species. Oecologia 105, 405-412.

Jakubowska, A.K., Vogel, H., Herrero, S., 2013. Increase in gut microbiota after immune suppression in baculovirus-infected larvae. PLoS Pathog. 9, e1003379.

Johansson, H., Dhaygude, K., Lindstrom, S., Helantera, H., Sundstrom, L., Trontti, K., 2013. A metatranscriptomic approach to the identification of microbiota associated with the ant *Formica exsecta*. PLoS One 8, e79777.

Lester, P.J., Buick, K.H., Baty, J.W., Felden, A., Haywood, J., 2019. Different bacterial and viral pathogens trigger distinct immune responses in a globally invasive ant. Sci. Rep. 9, 5780.

Lester, P.J., Sebastien, A., Suarez, A.V., Barbieri, R.F., Gruber, M.A., 2017. Symbiotic bacterial communities in ants are modified by invasion pathway bottlenecks and alter host behavior. Ecology 98, 861-874.

Lowe, S., Browne, M., Boudjelas, S., M., D.P., 2000. 100 of the world's worst invasive alien species: a selection from the global invasive species database. ISSG, New Zealand 12, 1-12.

Macom, T.E., Porter, S.D., 1996. Comparison of polygyne and monogyne red imported fire ant (Hymenoptera: Formicidae) population densities. Ann. Entomol. Soc. Am. 89, 535-543.

Riggs, N.L., Lennon, L., Barr, C.L., Drees, B.M., Cummings, S., Lard, C., 2002. Community-wide red imported fire ant management programs in Texas. Southwestern Entomologist Supplement 25, 31-41.

Sébastien, A., Lester, P.J., Hall, R.J., Wang, J., Moore, N.E., Gruber, M.A.M., 2015. Invasive ants carry novel viruses in their new range and form reservoirs for a honeybee pathogen. Biol. Lett. 11, 20150610.

Touyama, Y., Ogata, K., Sugiyama, T., 2003. The Argentine ant, *Linepithema humile*, in Japan: assessment of impact on species diversity of ant communities in urban environments. Entomol. Sci. 6, 57-62.

Valles, S.M., Strong, C.A., Dang, P.M., Hunter, W.B., Pereira, R.M., Oi, D.H., Shapiro, A.M., Williams, D.F., 2004. A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization. Virology 328, 151-157.

Wang, H., Meeus, I., Piot, N., Smagghe, G., 2018. Systemic Israeli acute paralysis virus (IAPV) infection in bumblebees (*Bombus terrestris*) through feeding and injection. J. Invertebr. Pathol. 151, 158-164.

Woolfolk, S., Stokes, C.E., Watson, C., Brown, R., Baird, R., 2016. Bacteria associated with red imported fire ants (*Solenopsis invicta*) from mounds in Mississippi. Southeast. Nat. 15, 83-101.

Wylie, R., Yang, C.C.S., Tsuji, K., 2020. Invader at the gate: the status of red imported fire ant in Australia and Asia. Ecol. Res. 35, 6-16.

Yang, C.C., Ascunce, M.S., Luo, L.Z., Shao, J.G., Shih, C.J., Shoemaker, D., 2012. Propagule pressure and colony social organization are associated with the successful invasion and rapid range expansion of fire ants in China. Mol. Ecol. 21, 817-833.

Yang, C.C., Yu, Y.C., Valles, S.M., Oi, D.H., Chen, Y.C., Shoemaker, D., Wu, W.J., Shih, C.J., 2010. Loss of microbial (pathogen) infections associated with recent invasions of the red imported fire ant *Solenopsis invicta*. Biol. Invasions 12, 3307-3318.

Chapter 2: Apoptosis as a primary defense mechanism in response to viral infection in invasive fire ant *Solenopsis invicta*

2.1 Introduction

Dicistroviridae is a viral family of positive-sense, single-strand RNA (ssRNA) viruses that infect arthropod hosts and are usually associated with an epidemic of chronic infections in the hosts, although dicistrovirus infections can vary considerably in virulence and pathogenicity (Bonning and Miller, 2010). Previous studies have demonstrated that dicistroviruses are associated with molting failure, reduced host longevity and fecundity, and even incur significant mortality in host populations (Bailey and Woods, 1977; Bekesi et al., 1999; Golubovsky and Plus, 1982; Williamson et al., 1988). With their host specificity and other traits (Lacey et al., 2015), some dicistroviruses have been considered potential biological control agents against invasive insect pests such as the red imported fire ant (*Solenopsis invicta*) and Argentine ant (*Linepithema humile*) (Gruber et al., 2017; Valles et al., 2018). As these viruses are currently being evaluated as self-sustaining biological control agents (Valles et al., 2018), knowledge regarding the interactions between them and their host ants represents baseline information to assure the control efficiency of any management framework that may be devised.

Solenopsis invicta virus 1 (SINV-1) is arguably the most well-studied ssRNA virus infecting ants (Valles et al., 2012). SINV-1 is found to mainly replicate at the alimentary canal of larvae in *S. invicta*, and brood death is occasionally found in SINV-1-infected colonies following translocation to the lab from the field (Valles et al., 2004; Valles et al., 2007). As late-instar larvae are generally more vulnerable to pathogen infections than other colony members due to the fact that they are the center of nutrient flow in the colony (i.e., they digest solid food particle for the entire colony) (Oi et al., 2005), their innate immune systems must have played a significant role in defending against SINV-1 and possibly other pathogens but remained overlooked.

The programmed cell death pathways (e.g., necrosis and apoptosis) are considered the intracellular innate immune mechanisms that restrict the dissemination of pathogens through directly destroying infected cells (Clarke and Clem, 2003; Nainu et al., 2015). In general, necrosis is a passive type of cell death caused by external factors, such as acute viral infection and cell destruction, and ends up with cell lysis, while apoptosis is commonly described as a non-inflammatory response of host against pathogen infection. The microsporidian pathogen of the genus *Nosema* in honeybees is recognized as an excellent model system to study the role of such defensive mechanisms in social insects. For example, Kurze et al. (2015) found that *N. ceranae* is able to manipulate cell apoptosis in *Nosema*-sensitive honeybees but has a negligible effect on tolerant honeybees, highlighting the immune function of apoptosis in honeybees when dealing with an intracellular gut pathogen such as microsporidium (Kurze et al., 2015). To our surprise, empirical studies reporting apoptosis as an immune pathway in virus-infected honeybees are lacking, not to mention studies on other groups of social insects such as ants. Although social insect colonies have evolved collective immune defenses against parasites and pathogens (Cremer et al., 2007), we argue that immune responses at the individual level (e.g., apoptosis) may also be important in preventing pathogen epidemics in the colony.

In this study, we therefore hypothesized that apoptosis in the midgut epithelium of the late-instar larvae of *S. invicta* is likely prevalent when challenged with SINV-1 infection. To test this hypothesis, we investigated the apoptotic status of 4th instar larval epithelia challenged by SINV-1 infection. To determine whether apoptosis in the midgut epithelium acts as a barrier to dissemination of SINV-1, we conducted a time-course study to monitor changes in both apoptosis signals and viral titers in the midgut epithelium of SINV-1-infected larvae. Results based on these two experiments are

expected to improve our understanding of the interplay between a viral pathogen and its social-insect host, but may also facilitate the development of future control framework on invasive ants.

2.2 Materials and methods

Colony collection and viral detection

Colonies of *S. invicta* (*N* = 40) were collected in northern Taiwan, and 10-15 workers were subject to molecular detection for the presence of numerous *S. invicta* viruses (SINV-1, 2 and 3) using species-specific RT-PCR (Hashimoto et al., 2007). SINV-1 and SINV-2 were detected in 60% (*N* = 24) and 25% (*N* = 10) of the collected colonies, respectively. As a result, a total of six colonies were confirmed to be uninfected and thus were maintained under the laboratory conditions for at least one month prior to the experiments.

Time course of viral replication

We isolated colony fragments, containing 10-20 4^{th} instar larvae and 200 workers, from each of the six uninfected colonies, and maintained the rest of the colonies for sampling of uninfected larvae as control. SINV-1-infected workers were homogenized and ultra-centrifuged by a sucrose density gradient for 2 hours at 350,000 rpm at 4 °C (Valles et al., 2004). Subsequently, the viral supernatants were quantified by real-time PCR and stored in -20 °C. Each fragment was then artificially inoculated with SINV-1 by feeding a viral suspension (10^9 genome equivalents/ml) mixed with 10% aqueous solution of honey for 24 hours after two days of starvation. One or two larvae (ten in total) were randomly taken from each of the six fragments for detection of viral amplification at four separate time points (1^{st}, 3^{rd}, 5^{th} and 7^{th} day after viral inoculation). Total RNA was extracted from an individual larva using TRIzol reagent (Invitrogen, CA, USA). cDNAs

with SINV-1 positive-sense were transcribed with a primer containing the 5' tag sequence GGCCGTCATGGTGGCGAA (Plaskon et al., 2009; Valles, 2012). Viral amplification was detected by strand-specific quantitative PCR in an ABI 7500 Real-Time PCR system following the protocol described by Hashimoto et al (Hashimoto et al., 2007). The correlation between inoculation period and viral titer was analyzed with a linear mixed model with the colony as the random effect. The effect of inoculation period was examined by likelihood ratio test by comparing the full model and the model without it as the fixed effect. The models were built by "lme4" package in R (R Development Core Team, 2014) with the function, "lmer".

Apoptosis detection in the larval midgut

One or two larvae (ten in total) were collected from each of the six artificially infected fragments at four time points and were then subject to TUNEL assay to monitor the apoptosis level, whereas larvae of the same instar also were sampled from the original uninfected colonies as a control group. TUNEL assay is commonly used to detect the DNA fragmentation, a key feature of apoptosis, by staining terminal deoxynucleotidyl transferase (TdT) that catalyzes attachment of modified deoxynucleotides on the DNA strand breaks during the late stage of apoptotic pathway (Darzynkiewicz and Zhao, 2011). Both uninfected and infected larvae were soaked in 20% sucrose for 1 hour to avoid tissue damage and were then embedded in Tissue-Tek® O.C.T.™ Compound before being transferred to the cryomolds. The cryo-sections (30 μm) were mounted on the poly-L-lysine-coated glass slides and fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min at room temperature. The slides were processed and stained by TdT according the protocol of an *in situ* Apoptosis Detection Kit (TaKaRa, Japan). The apoptotic signals labeled with fluorescence-dUTP were monitored by fluorescence microscopy with a 20× fluorescent objective. We focused on the larval

midgut for detecting the signal of apoptosis because replication of SINV-1 has been reported to center in this particular part of the alimentary canal (Valles, 2012). We measured the TdT-positive area in the midgut tissue in each tissue section using ImageJ (NIH, imagej.nih.gov), and estimated apoptosis rate by dividing the TdT-positive area by the total area of midgut (apoptosis rate = TdT-positive area/total area of midgut). The effect of time elapsed since inoculation on apoptotic status was analyzed using the generalized linear mixed model with binomial errors and colony as random effects. The significance of the effect was examined by the likelihood ratio test by comparing the full model and the model without it as the fixed effect. The models were built by "lme4" package with the function, "glmer".

Ultrastructural examination

To understand the morphological alteration associated with SINV-1 in the midgut epithelium, we collected 4th instar larvae from uninfected colonies and infected fragments of the same colonies on the 7th day after viral inoculation. The fire ant larvae were dissected and fixed overnight with 1% glutaraldehyde in 0.05-M cacodylate buffer (pH = 7.0), and secondarily fixed with 1% 0.05-M OsO_4 solution in cacodylate buffer for 1 hour. The fixed tissues were then dehydrated through serial acetone. The larvae were embedded in Spurr resin (Spurr, 1969), and tissues were identified in 1-μm-thick sections using Toluidine blue O stains (TBO). Ultra-thin (100 nm) sections were stained with uranyl acetate and lead citrate and visualized under transmission electron microscopy (Hitachi H-7650, TC5 Bio-image Tools, NTU).

2.3 Results

We characterized host-pathogen dynamics by examining changes of viral replication at four time points after infection. Results indicated a trend of viral amplification reduction with the time elapsed since inoculation; a significant negative correlation was

found between the two parameters (*p*-value <0.001) (Figure 2.1a, Table 2.1). As detected by TUNEL assay, only a few apoptotic cells were found in the midgut of the uninfected larvae and infected larvae during the early viral inoculation (the 1st and 3rd day). Apoptotic cells, however, were abundant in the midgut epithelium of the infected larvae on the 5th day and reached a peak on the 7th day after inoculation during our experimental period (Figure 2.1b, c). The delayed increase in apoptotic level may simply reflect the nature of TUNEL assay as DNA fragmentation is a late-stage event during the apoptotic progression (Collins et al., 1997; Wadskog et al., 2004). The intensity, as expressed by apoptosis rate, was positively correlated with the time elapsed since initial inoculation (*p*-value <0.001) (Figure 2.1b, Table 2.1).

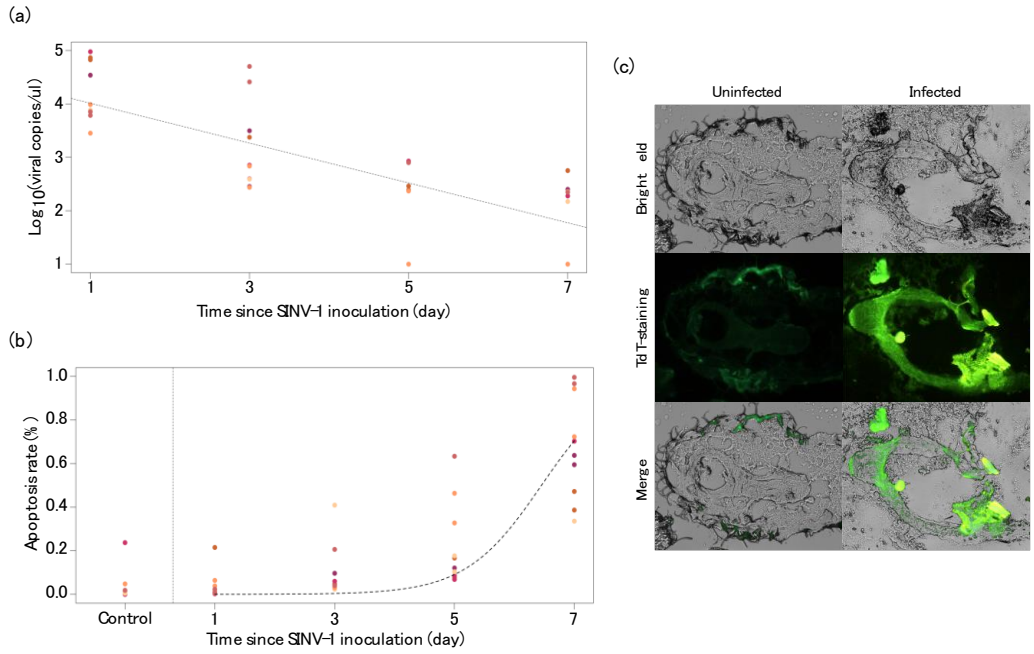

Figure 2.1 A negative correlation between viral titer and apoptotic level during the first 7 days after artificial SINV-1 infection. a) viral titers across four time points; b) apoptosis rates across four time points; c) the proportions of TdT-positive tissue in the midgut epithelium by TUNEL assay in uninfected and infected larvae (original magnification: 20×). Note that a similarly low apoptosis level is observed among all uninfected larvae, and hence we only present the apoptosis level from an individual in (c).

Table 2.1 The significances of "viral titer" and "apoptotic level" after viral inoculation

Source	β_0	β_1	*d.f.*	X^2	P
Viral titer	3.387	-0.373	1	37.127	<0.001
Apoptotic level	-10.224	1.585	1	21.215	<0.001

The microscopy examination showed that the midgut epithelium of uninfected larvae remains functionally active as evidenced by abundant lipid droplets and cell organelles such as mitochondria and endoplasmic reticulum (Figure 2.2a-d). Furthermore, the microvilli-covered apical membrane contained bundles of filaments, suggesting maintenance of the functions and properties of the midgut epithelium (Figure 2.2a). Unlike uninfected larvae, numerous apoptotic cells were found in midgut epithelium of infected larvae (Figure 2.2e). The ultrastructure of the midgut epithelium in the infected larvae was characterized by chromatin condensation and reduction of the number of the microvilli; in addition, quite a few cytoplasmic vacuoles were present at the base, suggesting that the cells had undergone a series of degradation processes (Figure 2.2a, b). Numerous midgut epithelial cells in SINV-1-infected larvae were found to have swollen cytoplasm, nuclear fragmentation and condensed chromatin (Figure 2.2e, f). Furthermore, reduced numbers of organelles and less blank space in some epithelial cells were observed after infection (Figure 2.2e, f). We found plenty of cytoplasmic vacuoles rather than organelles in the infected larvae (Figure 2.2f). Moreover, the structural disorganization and dilated intercristal space suggested mitochondria were in the early stages of degeneration (Figure 2.2g). The formation of apoptotic bodies in a midgut epithelial cell was observed in the late stages of apoptosis (Figure 2.2h). On the contrary, few epithelial cells, if any, were observed to be undergoing apoptosis in the midgut epithelium of uninfected larvae. Hence, the presence of apoptotic cells in the midgut epithelium was confirmed to be a response to the challenge by SINV-1.

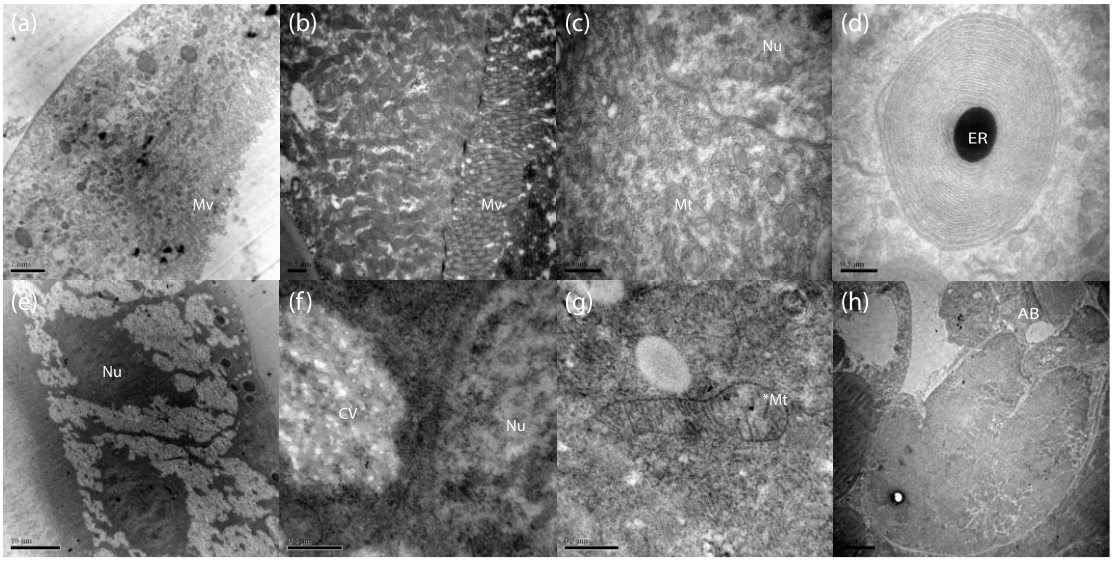

Figure 2.2 Ultrastructure features of the larval midgut epithelial cells collected from uninfected colonies [a-d] and infected colonies [e-h]: (a) epithelial cells filled with organelles; (b) microvilli are abundant in the epithelial cells; (c) numerous mitochondria observed in a healthy epithelial cell; (d) normal structure of the endoplasmic reticulum; (e) nuclear condensation; (f) reduction of organelles and formation of cytoplasmic vacuoles; (g) structural disorganization and dilated intracristal space of mitochondria; (h) the formation of apoptotic bodies. [Note: AB, apoptotic bodies; CV, cytoplasmic vacuoles; ER, endoplasmic reticulum. Mt, mitochondria; Mv, microvilli; Nu, nucleus]

2.4 Discussion

While viruses are considered potential biological control agents against invasive fire ants (Valles et al., 2018), studies towards a better understanding of the complex interactions between microbe and host ant are much needed to assure control efficiency. This study demonstrated that SINV-1 infection induces apoptosis in the midgut epithelium of fire ants and that the viral replication of SINV-1 decreases as the number of apoptotic cells increases. These data are consistent with previously observed tissue tropisms for SINV-1 (Hashimoto and Valles, 2007) and other insect-infecting positive-sense, ssRNA viruses (Valles, 2012). These findings also suggest that apoptosis of midgut epithelium cells in fire ant larvae likely serves as a barrier to prevent SINV-1

dissemination, which in turn may explain the low virulence of SINV-1 in fire ants. However, it remains unclear how a high SINV-1 titer might be retained in field colonies of fire ants, as has been observed in some cases (Hashimoto and Valles, 2007). One possibility is that apoptosis is only effective in the containment of viral dissemination when the viral titer is below a certain threshold; indeed, apoptosis is considered a threshold-dependent response (Fragkoudis et al., 2009). In this study, we fed the experimental colonies with a fixed viral dose only at the beginning of the experiment, with only a 24-hr inoculation period; hence, the titer may well have been sub-threshold.

While our results showed that an apoptotic midgut epithelium may function as a potentially effective defensive mechanism against SINV-1, significant brood mortality has been observed in SINV-1-infected colonies, particularly when colonies are challenged with certain stressful conditions (e.g., colony translocation) (Valles et al., 2004). Many arthropod-infecting positive-sense, ssRNA viruses share a similar chronic, asymptomatic infection nature but cause overt symptoms under certain circumstances including rapid viral replication under environmental stress triggers (Chen and Siede, 2007; de Miranda and Genersch, 2010), and SINV-1 seems to be no exception (Valles, 2012). This possibility is supported by multiple recent studies in which widespread apoptosis in response to pathogen challenge resulted in increasing mortality of various insect hosts due to overwhelming immunopathology (Wang et al., 2012). Studies examining the interactions of viral titer, environmental stress, apoptosis level and colony mortality are needed in the future.

Recent evidence indicates that SINV-1 infection alters the food preference of fire ants towards carbohydrate-rich foods compared to uninfected congeners (Hsu et al., 2018; Valles et al., 2013; Valles et al., 2014), which is believed to prefer a high-protein, high-lipid diet (Stein et al., 1990; Vander Meer et al., 1995). Late-instar larvae in fire ants

are the primary colony members that digest solid food and redistribute the food through trophallaxis in liquid form to nestmates (Cassill and Tschinkel, 1999), and it is likely that an increased level of epithelial apoptosis as result of defending against SINV-1 infection may impair the digestive capabilities of these larvae (Hausmann, 2010; Schafer et al., 2013; Sonakowska et al., 2016), driving workers to forage only liquid foods such as honey solution in response to the larval incompetence. Furthermore, larval conditions in a colony play a critical role in regulating the ontogeny of workers' forging behaviors (Ulrich et al., 2016). This may also explain why SINV-1-infected fire ants forage much less, another key reported behavioral change induced by SINV-1 infection (Hsu et al., 2018). Hence, the reduced foraging performance of workers may simply reflect larval status (e.g., loss of appetite) associated with widespread SINV-1-induced apoptosis in the intestinal epithelium. The results of TEM examination appear to support this possibility given the fact that the morphology of the midgut epithelium in infected larvae is significantly altered with apparent reduction of organelles and nuclear deformation, which likely reduces the functions of food processing (e.g., secretion of digestive enzymes) and thus digestion competence in SINV-1-infected larvae (Huang et al., 2015).

Accumulating evidence in other species has shown that pathogen-induced apoptosis may potentially incur fitness costs (Medzhitov et al., 2012; Sonakowska et al., 2016), leading researchers to suggest disease tolerance as an alternative, less costly defensive mechanism in coping with infection through anorexia or dietary changes (e.g., compensatory feeding) (Abbott, 2014; Medzhitov et al., 2012; Povey et al., 2014; Shikano and Cory, 2016). SINV-1 in fire ants seems to be an excellent model system for such studies, as similar illness behaviors including reduced foraging activities (analogical to anorexia) and shifts in macronutrient preference have been reported in

SINV-1-infected fire ants (Hsu et al., 2018). Furthermore, the host-virus interactions during early (acute) and late (chronic) infection phases may involve different immune cascades, host immune responses and gene expression profiles (Belov et al., 2003; Buenz and Howe, 2006; Croft et al., 2017). Thus, further studies on SINV-1 and fire ants would facilitate our understanding of early-late switch temporal regulations associated with a focal virus, and provide useful knowledge for integrating viruses into current control schemes for fire ants.

2.5 References

Abbott, J., 2014. Self-medication in insects: current evidence and future perspectives. Ecol. Entomol. 39, 273-280.

Bailey, L., Woods, R.D., 1977. Two more small RNA viruses from honey bees and further observations on sacbrood and acute bee-paralysis viruses. J. Gen. Virol. 37, 175-182.

Bekesi, L., Ball, B.V., Dobos-Kovacs, M., Bakonyi, T., Rusvai, M., 1999. Occurrence of acute paralysis virus of the honey bee (*Apis mellifera*) in a Hungarian apiary infested with the parasitic mite *Varroa jacobsoni*. Acta. Vet. Hung. 47, 319-324.

Belov, G.A., Romanova, L.I., Tolskaya, E.A., Kolesnikova, M.S., Lazebnik, Y.A., Agol, V.I., 2003. The major apoptotic pathway activated and suppressed by poliovirus. J. Virol. 77, 45-56.

Bonning, B.C., Miller, W.A., 2010. Dicistroviruses. Annu. Rev. Entomol. 55, 129-150.

Buenz, E.J., Howe, C.L., 2006. Picornaviruses and cell death. Trends Microbiol. 14, 28-36.

Cassill, D.L., Tschinkel, W.R., 1999. Regulation of diet in the fire ant, *Solenopsis invicta*. J. Insect Behav. 12, 307-328.

Chen, Y.P., Siede, R., 2007. Honey bee viruses. Adv. Virus Res. 70, 33-80.

Clarke, T.E., Clem, R.J., 2003. Insect defenses against virus infection: the role of apoptosis. Int. Rev. Immunol. 22, 401-424.

Collins, J.A., Schandl, C.A., Young, K.K., Vesely, J., Willingham, M.C., 1997. Major DNA fragmentation is a late event in apoptosis. J. Histochem. Cytochem. 45, 923-934.

Cremer, S., Armitage, S.A., Schmid-Hempel, P., 2007. Social immunity. Curr. Biol. 17, R693-702.

Croft, S.N., Walker, E.J., Ghildyal, R., 2017. Picornaviruses and apoptosis: subversion of cell death. mBio. 8.

Darzynkiewicz, Z., Zhao, H., 2011. Detection of DNA strand breaks in apoptotic cells by flow- and image-cytometry. Methods Mol. Biol. 682, 91-101.

de Miranda, J.R., Genersch, E., 2010. Deformed wing virus. J. Invertebr. Pathol. 103, 48-61.

Fragkoudis, R., Attarzadeh-Yazdi, G., Nash, A.A., Fazakerley, J.K., Kohl, A., 2009. Advances in dissecting mosquito innate immune responses to arbovirus infection. J. Gen. Virol. 90, 2061-2072.

Golubovsky, M.D., Plus, N., 1982. Mutability studies in two *Drosophila melanogaster* isogenic stocks, endemic for C picornavirus and virus-free. Mutat. Res. 103, 29-32.

Gruber, M.A.M., Cooling, M., Baty, J.W., Buckley, K., Friedlander, A., Quinn, O., Russell, J., Sebastien, A., Lester, P.J., 2017. Single-stranded RNA viruses infecting the invasive Argentine ant, *Linepithema humile*. Sci. Rep. 7, 3304.

Hashimoto, Y., Valles, S.M., 2007. *Solenopsis invicta* virus-1 tissue tropism and intra-colony infection rate in the red imported fire ant: a quantitative PCR-based study. J. Invertebr. Pathol. 96, 156-161.

Hashimoto, Y., Valles, S.M., Strong, C.A., 2007. Detection and quantitation of *Solenopsis invicta* virus in fire ants by real-time PCR. J. Virol. Methods 140, 132-139.

Hausmann, M., 2010. How bacteria-induced apoptosis of intestinal epithelial cells contributes to mucosal inflammation. Int. J. Inflam. 2010, 574568.

Hsu, H.W., Chiu, M.C., Shoemaker, D., Yang, C.S., 2018. Viral infections in fire ants lead to reduced foraging activity and dietary changes. Sci. Rep. 8, 13498.

Huang, J.H., Jing, X., Douglas, A.E., 2015. The multi-tasking gut epithelium of insects. Insect Biochem. Mol. Biol. 67, 15-20.

Kurze, C., Le Conte, Y., Dussaubat, C., Erler, S., Kryger, P., Lewkowski, O., Muller, T., Widder, M., Moritz, R.F., 2015. Nosema tolerant honeybees (*Apis mellifera*) escape parasitic manipulation of apoptosis. PLoS One 10, e0140174.

Lacey, L.A., Grzywacz, D., Shapiro-Ilan, D.I., Frutos, R., Brownbridge, M., Goettel, M.S., 2015. Insect pathogens as biological control agents: back to the future. J. Invertebr. Pathol. 132, 1-41.

Medzhitov, R., Schneider, D.S., Soares, M.P., 2012. Disease tolerance as a defense strategy. Science 335, 936-941.

Nainu, F., Tanaka, Y., Shiratsuchi, A., Nakanishi, Y., 2015. Protection of insects against viral infection by apoptosis-dependent phagocytosis. J. Immunol. 195, 5696-5706.

Oi, D.H., Briano, J.A., Valles, S.M., Williams, D.F., 2005. Transmission of *Vairimorpha invictae* (Microsporidia: Burenellidae) infections between red imported fire ant (Hymenoptera: Formicidae) colonies. J. Invertebr. Pathol. 88, 108-115.

Plaskon, N.E., Adelman, Z.N., Myles, K.M., 2009. Accurate strand-specific quantification of viral RNA. PLoS One 4, e7468.

Povey, S., Cotter, S.C., Simpson, S.J., Wilson, K., 2014. Dynamics of macronutrient self-medication and illness-induced anorexia in virally infected insects. J. Anim. Ecol. 83, 245-255.

Schafer, C., Schroder, K.R., Hoglinger, O., Tollabimazraehno, S., Lornejad-Schafer, M.R., 2013. Acetaminophen changes intestinal epithelial cell membrane properties, subsequently affecting absorption processes. Cell Physiol. Biochem. 32, 431-447.

Shikano, I., Cory, J.S., 2016. Altered nutrient intake by baculovirus-challenged insects: self-medication or compensatory feeding? J. Invertebr. Pathol. 139, 25-33.

Sonakowska, L., Wlodarczyk, A., Wilczek, G., Wilczek, P., Student, S., Rost-Roszkowska, M.M., 2016. Cell death in the epithelia of the intestine and hepatopancreas in *Neocaridina heteropoda* (Crustacea, Malacostraca). PLoS One 11, e0147582.

Spurr, A.R., 1969. A low-viscosity epoxy resin embedding medium for electron microscopy. J. Ultrastruct Res. 26, 31-43.

Stein, M.B., Thorvilson, H.G., Johnson, J.W., 1990. Seasonal changes in bait preference by red imported fire ant, *Solenopsis invicta* (Hymenoptera, Formicidae). Fla. Entomol. 73, 117-123.

Ulrich, Y., Burns, D., Libbrecht, R., Kronauer, D.J., 2016. Ant larvae regulate worker foraging behavior and ovarian activity in a dose-dependent manner. Behav. Ecol. Sociobiol. 70, 1011-1018.

Valles, S.M., 2012. Positive-strand RNA viruses infecting the red imported fire ant, *Solenopsis invicta*. Psyche 2012, 1-14.

Valles, S.M., Oi, D.H., Yu, F., Tan, X.X., Buss, E.A., 2012. Metatranscriptomics and pyrosequencing facilitate discovery of potential viral natural enemies of the invasive Caribbean crazy ant, *Nylanderia pubens*. PLoS One 7, e31828.

Valles, S.M., Porter, S.D., Calcaterra, L.A., 2018. Prospecting for viral natural enemies of the fire ant *Solenopsis invicta* in Argentina. PLoS One 13, e0192377.

Valles, S.M., Porter, S.D., Choi, M.Y., Oi, D.H., 2013. Successful transmission of *Solenopsis invicta* virus 3 to *Solenopsis invicta* fire ant colonies in oil, sugar, and cricket bait formulations. J. Invertebr. Pathol. 113, 198-204.

Valles, S.M., Porter, S.D., Firth, A.E., 2014. *Solenopsis invicta* virus 3: pathogenesis and stage specificity in red imported fire ants. Virology 460, 66-71.

Valles, S.M., Strong, C.A., Dang, P.M., Hunter, W.B., Pereira, R.M., Oi, D.H., Shapiro, A.M., Williams, D.F., 2004. A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization. Virology 328, 151-157.

Valles, S.M., Strong, C.A., Oi, D.H., Porter, S.D., Pereira, R.M., Vander Meer, R.K., Hashimoto, Y., Hooper-Bui, L.M., Sanchez-Arroyo, H., Davis, T., Karpakakunjaram, V., Vail, K.M., Fudd Graham, L.C., Briano, J.A., Calcaterra, L.A., Gilbert, L.E., Ward, R., Ward, K., Oliver, J.B., Taniguchi, G., Thompson, D.C., 2007. Phenology, distribution, and host specificity of *Solenopsis invicta* virus-1. J. Invertebr. Pathol. 96, 18-27.

Vander Meer, R.K., Lofgren, C.S., Seawright, J.A., 1995. Specificity of the red imported fire ant (Hymenoptera, Formicidae) phagostimulant response to carbohydrates. Fla. Entomol. 78, 144-154.

Wadskog, I., Maldener, C., Proksch, A., Madeo, F., Adler, L., 2004. Yeast lacking the SRO7/SOP1-encoded tumor suppressor homologue show increased susceptibility

to apoptosis-like cell death on exposure to NaCl stress. Mol. Biol. Cell 15, 1436-1444.

Wang, H., Gort, T., Boyle, D.L., Clem, R.J., 2012. Effects of manipulating apoptosis on Sindbis virus infection of *Aedes aegypti* mosquitoes. J. Virol. 86, 6546-6554.

Williamson, C., Rybicki, E.P., Kasdorf, G.G.F., Vonwechmar, M.B., 1988. characterization of a new picorna-like virus isolated from aphids. J. Gen. Virol. 69, 787-795.

Chapter 3: Viral infections lead to reduced foraging activity and dietary changes in invasive fire ant *Solenopsis invicta*

3.1 Introduction

Social insects have evolved sophisticated defensive systems presumably as adaptive responses to pathogens commonly encountered because of frequent social contact. Both allo-grooming and secretion of antibiotic compounds are two often cited examples reported in social insects (Simone-Finstrom, 2017; Tragust et al., 2013; Ugelvig and Cremer, 2007; Zeng Y, 2014). Conversely, some pathogens have been reported to alter host behavior to favor their own transmission and enhance their replication and virulence (Hafer, 2016). Studies of non-social insects have reported reduced feeding (anorexia) and changes in dietary macronutrient preference in pathogen-challenged individuals, both of which are associated with an enhanced ability to cope with pathogen infections likely via starvation of resident pathogens from essential macronutrients or trade-offs in energy allocation (Mason et al., 2014). Whether similar defensive mechanisms involving illness-induced behavioral changes also occur in social insects, especially ants, remain ambiguous (Adamo et al., 2010; Singer et al., 2014).

In this study, we tested whether reduced foraging activity or changes in macronutrient preference occur in the highly social fire ant *Solenopsis invicta* in response to a natural viral pathogen, *Solenopsis invicta* virus 1 (SINV-1). This virus is a positive-sense, single-stranded RNA (ssRNA) virus that apparently only infects ant species in the genus *Solenopsis* (Valles, 2012). This virus is believed to persist largely as a chronic and asymptomatic infection but may become virulent and result in illness and colony death when ant colonies are stressed (Valles and Hashimoto, 2009). More specifically, recent studies demonstrate this virus leads to high larval mortality under certain laboratory rearing conditions and significant weight loss of queens during colony founding (Manfredini et al., 2016). Several immune-related genes are up-regulated in

SINV-1-infected fire ants (Manfredini et al., 2016), further suggesting the virus imposes significant fitness costs under certain stressful conditions.

The discovery of SINV-1 in invasive populations of *S. invicta* (e.g., Taiwan (Yang et al., 2010)) offers an excellent opportunity to investigate the role of pathogen-induced behaviors in colony survival of *S. invicta*, especially during the initial colony founding stage when queens are known to experience significant stress as a result of starvation and exposure to harsh environmental conditions. *S. invicta* are opportunistic omnivores that feed on a wide variety of food resources including arthropod preys, hemipteran honeydew and plant materials, etc (Glunn et al., 1981; Stein et al., 1990). Despite great variations in their dietary preference, *S. invicta* have been generally regarded as oil-loving ants (Stein et al., 1990; Vander Meer et al., 1995), and, based on such behavior, oil has been impregnated in conventional bait products a phagostimulant to stimulate stronger feeding/foraging activity of fire ants and thus to achieve proper control (Vander Meer et al., 1995). Since knowledge on interplay among feeding stimulant, foraging patterns and dietary preference plays a critical role in success of bait-based management in fire ants (Hooper-Bui et al., 2002), demonstration that SINV-1 induces reduced foraging activity or changes in macronutrient intake are of critical importance because alterations of feeding rates and dietary preferences may impact the efficacies of poison baits used for fire ant control and population monitoring methods.

3.2 Materials and methods

Colony identification, fragment formation and virus inoculations

Fire ant colonies were collected from northern Taiwan. The social form and infection status of colonies with respect to three *S. invicta* viruses (namely SINV-1, SINV-2 and SINV-3) were determined using diagnostic PCR assays described in detail elsewhere (Valles and Porter, 2003; Valles et al., 2009). Colonies were maintained at standard

rearing conditions of 28 ±1°C, 70 ± 10% RH and a photoperiod of 12:12 (L:D). Virus-free monogyne colonies (n=9) were separated into two colony fragments, each consisting of 3g of workers (nearly 3,000 individuals) and 0.02g of brood. Note that polygyne colonies were not included in this study due to scarcity of virus-free colonies of this given social form in the field (Allen et al., 2011; Yang et al., 2010). Fragments were maintained on a diet of frozen crickets, sucrose solution and water. The single mother queen was randomly assigned to one of the two fragments (Appendix 1) as the presence of queen may play a role in shaping colony foraging motivation (Manfredini et al., 2014). Equivalent viral titers (10^9 genome equivalents/ml) of SINV-1 were artificially inoculated into one of each pair of colony fragments. Inoculations were done via feeding viral suspension mixed with 10% aqueous solution of honey for 24 hours (Valles et al., 2004). Behavioral assays were conducted 30 days post inoculation. SINV-1 infection status of each fragment was monitored using RT-PCR every week after the inoculation until the end of the experiments.

Behavioral assays: foraging patterns and changes in food preference

Different food resources with varying macronutrients, which are commonly used for monitoring of ant diversity in the field (Nyamukondiwa and Addison, 2014; Stringer et al., 2011), were provided to each colony fragment to examine the effects of SINV-1 infections on ant foraging patterns and macronutrient preferences (Appendix 2). Each food resource was placed in a single container coated with fluon and connected independently to the colony fragment using a plastic tube (1m). To quantify the foraging intensity, we recorded total numbers of foragers at all four food resources using an automatic recording system (camera Olympus TG-3, Olympus Corp., Japan) that takes a picture every 10 minutes for 32 hours. These pictures were analyzed to estimate the total number of foraging workers at each time point using a naked-eye-

counting strategy. Time required for the first five workers to arrive at one of the four food resources was measured (onset of foraging). We also measured recruitment efficiency, defined as the time elapsed until peak in foraging was reached during a 32-hour observation period. Food preference was determined by assessing the number of workers at each food resource in both uninfected and infected fragments.

Statistical analyses

All the colony fragments (a total of 18 fragments from 9 colonies) were divided into one of four categories based on infection status/presence of queen: uninfected w/ queen (n = 4), uninfected w/o queen (n = 5), infected w/ queen (n = 5), and infected w/o queen (n = 4). The effects of viral infection on fire ant foraging behavior were analyzed by comparing foraging patterns and food preferences between uninfected and SINV1-infected fragments.

For the statistical analysis of foraging intensity, defined as total number of forager workers observed on all four food resources, the effects of infection and the presence of queen were modeled by using generalized linear mixed models (GLMMs) with Poisson errors and negative-binomial errors. Despite nearly identical results, we only showed the results estimated with negative-binomial errors because of lower Akaike information criterion (AIC) values and overdispersion (Chi-square test). The numbers of ants at each time point are considered as response variables, infection status and presence of queen as fixed effects, and colony identity and each time point as random factors. The full model was built by "lme4" package in *R* (R Development Core Team, 2014) with the function, "glmer". Significances of the fixed effects were tested using the likelihood ratio test by comparing the full model and models sequentially removing each effect term. The post hoc multiple comparisons of the four fragment categories was conducted with Tukey's all-pairwise comparisons implemented in the *R* package

"multcomp". The effects of viral infection on foraging onset and recruitment efficiency were analyzed by Pearson chi-square test (*R* package: "nortest"). The significances among the four fragment categories were first analyzed by a one-way ANOVA or Kruskal-Wallis test, and then post hoc by pairwise *T* test or pairwise Kruskal-Wallis test with the Bonferroni correction. All the values were shown by mean ± SD.

For changes in food preference, the full mixed model was built using negative-binomial errors with the total ant numbers at each time point considered as response variables, and colony identity and each time point as random factors. Food types were set as dummy factors, and the interaction effects of food types and infection status or the presence of queen were analyzed using the function "glmer" implemented in "lme4" package (R Development Core Team, 2014). The likelihood ratio test was used to determine significance level of each fixed effect by reducing the full model. The relationship between food preference and fragment categories was analyzed using a local regression (locally weighted scatterplot smoothing curve, LOWESS curve). Food preference was ranked, and the significances for each pairwise comparison of the four food resources were determined using the Tukey's honesty significant difference (HSD) test.

3.3 Results

Results from our behavioral assays revealed significant differences in foraging patterns and macronutrient preferences between the uninfected and SINV-1-infected colony fragments. While the absence of a queen is associated with reduced foraging performance in this study, the impacts of viral infection outweigh the effects associated with queen presence and play a critical role in regulating foraging patterns of *S. invicta*, especially foraging intensity and recruitment efficiency.

Numbers of workers foraging on food resources were higher in uninfected fragments compared with SINV-1-infected fragments. Both SINV-1 infection and the absence of a queen significantly reduced the total number of forager workers, but the former had a greater impact (Figure 3.1; Table 3.1). A similar pattern was observed for recruitment efficiency. Uninfected fragments reached peak foraging activities significantly faster than SINV-1-infected fragments. The time required to reach foraging peak significantly differed among the four fragment categories (Figure 3.1, Appendix 3, ANOVA: F = 21.96, df = 3, $P < 0.001$). The time required for foraging onset was not significantly different among fragment categories (Appendix 3, Kruskal–Wallis test: X^2 = 5.00, df = 3, P = 0.1716).

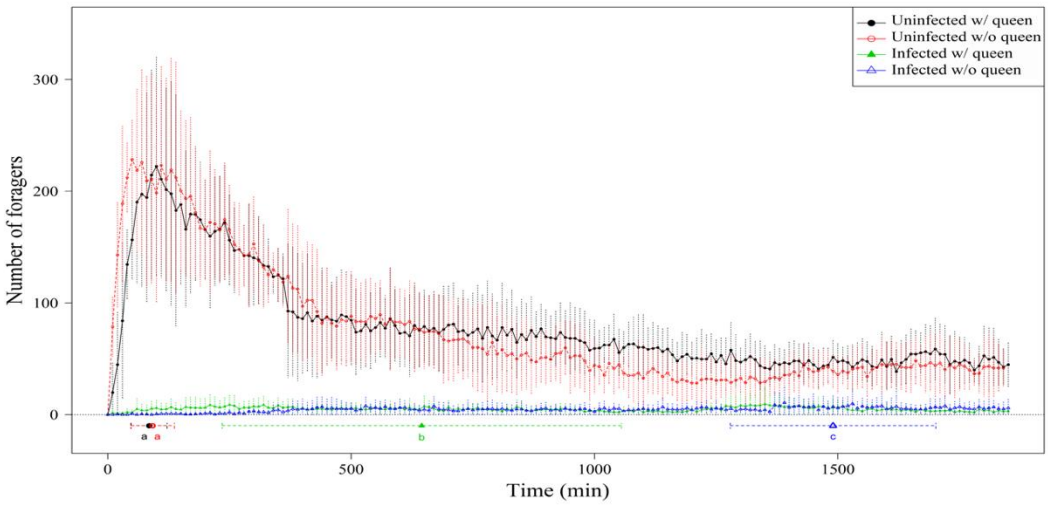

Figure 3.1 Foraging activities and peak recruitment time of uninfected (round) and SINV-1-infected (triangle) colony fragments. The open and closed symbols denote the presence and absence of a queen in a fragment, respectively. The letters (a-c) indicate significant pairwise differences (multiple comparisons conducted using pairwise T test, P < 0.05) across the four fragment categories.

Workers began to forage as soon as 17.5±9.57 mins after being offered food resources in uninfected colony fragments with a queen. The recruitment efficiency in these fragments peaked at 85±39.79 mins. Approximately 400 mins after food offered, the

numbers of the workers decreased and stabilized at ca. 50% of the peak number (Figure 3.1, Appendix 3). Uninfected colony fragments lacking a queen displayed similar patterns in both onset of foraging (18.00±13.04 mins) and recruitment efficiency (92.00±44.94 mins). Queen presence had no significant effect on recruitment efficiency in uninfected fragments (Figure 3.1, Appendix 3).

Workers from SINV-1-infected fragments showed significant differences in foraging intensity compared with uninfected workers. The number of foraging workers from the former was significantly lower than that from uninfected fragments (presence or absence of a queen had no effect) (Appendix 3). The onset of foraging in SINV-1-infected fragments also was delayed (310±504.58 mins) but not statistically significant from the other three fragment categories. SINV-1-infected fragments reached a peak in foraging significantly later than uninfected fragments (622±501.47 mins) (Figure 3.1). We detected no effect of queen presence on the number of workers or the time of the foraging onset (135.00±113.58 mins with queen; 1452±169.39 mins without queen) in SINV-1-infected fragments.

Analyses of numbers of workers on different food resources indicated workers from uninfected fragments prefer high lipid or protein content food (i.e., tuna and peanut butter). In contrast, workers from SINV-1-infected fragments significantly preferred diluted honey or potato chip compared with those from uninfected fragments (Figure 3.2; Table 3.1). Moreover, the presence of a queen influenced colony food preferences. All uninfected queenright fragments significantly preferred tuna, a food resource with relatively higher lipid and protein content (Figure 3.2; Table 3.1).

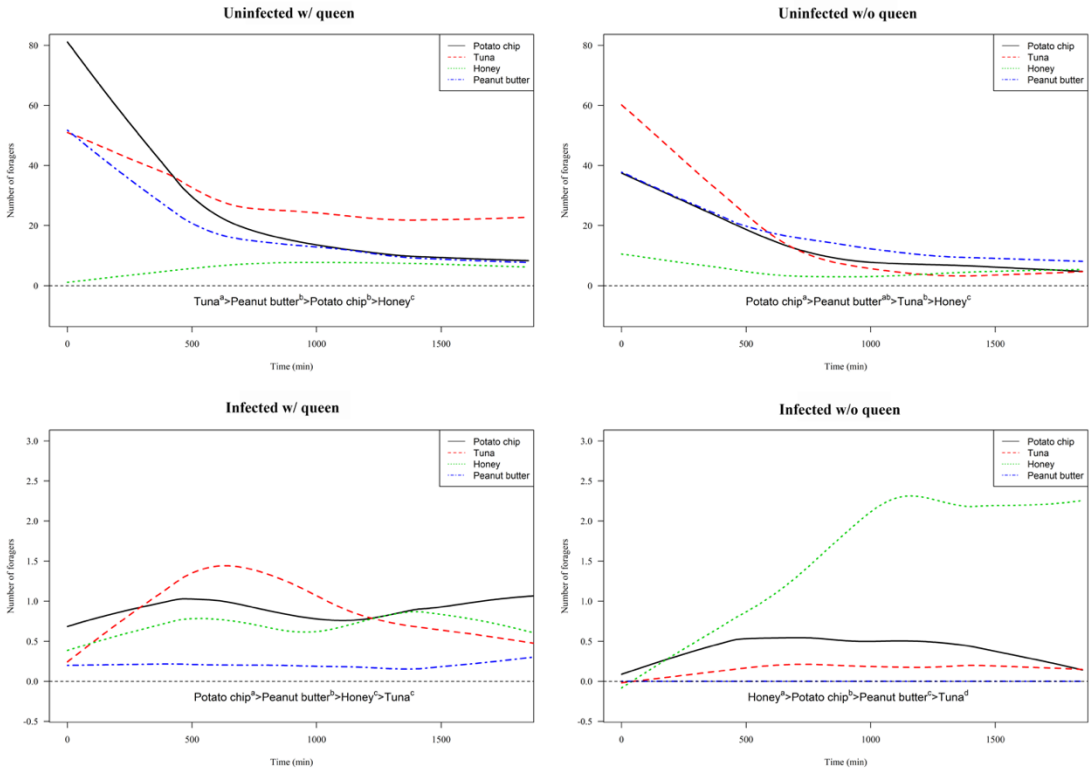

Figure 3.2 Food preference expressed as the number of workers at each food resource across time. Letters (a-d) indicate significant pairwise differences among each of the four food types across the four fragment categories (multiple comparisons conducted using a Tukey's honestly significant difference test under generalized linear model, $P < 0.05$). Note quantitative scales for the y-axis differ among the graphs due to differences in number of foragers between uninfected and infected fragments.

Table 3.1 Fixed effects of "virus infection" and "presence of the queen" on foraging behaviors

Source	d.f.	X^2	P
Foraging intensity			
Queen	2	52.007	<0.001***
Infection	2	3507.8	<0.001***
Queen × Infection	1	0.131	0.7174
Food preference			
Food type	3	120.99	<0.001***
Queen × Food type	4	340.23	<0.001***
Infection × Food type	4	7022.2	<0.001***

3.4 Discussion

Our results provide evidence for both reduced foraging activity and a change in macronutrient preferences of virus-challenged fire ants. SINV-1-infected ants invariably displayed reduced foraging activities compared with uninfected ants. SINV-1-infected ants also altered their feeding preferences toward a carbohydrate-rich diet. Similar findings were reported in fire ants infected with another single strand-RNA virus (SINV-3) (Valles et al., 2013; Valles et al., 2014), suggesting such behavioral alterations might be common in the ant host challenged with a viral infection. Potential adaptive advantages and the illness consequences of these observed virus-induced changes in foraging behavior are discussed below, followed by the potential management implications of our results.

SINV-1 replicates in the gut epithelium of *S. invicta* and spreads among nestmates largely through trophallaxis (Valles and Hashimoto, 2009). Reductions in foraging activity of infected workers may lead to decreases in intra-colony food transfer and exchange, which in turn could reduce SINV-1 transmission among nestmates. Reduced social interactions as a result of decreased foraging activity have been documented in honey bees (Kazlauskas et al., 2016; Schmid-Hempel, 2017). For example, injection of lipopolysaccharides into honey bees resulted in appetite loss and a reduction in social interactions, and both of these behavioral changes may contribute to reducing potential pathogen transmission. While such behavioral changes are due to direct virus-induced fitness costs or tradeoffs between immune response and energy allocation remains unclear, reduced social interactions and illness-induced anorexia in diseased colonies could be advantageous (e.g., less trophallaxis and brood tending) by reducing potential for spread of infections (Bos et al., 2012; Ugelvig and Cremer, 2007). We suggest that the fire ant-virus system is an ideal candidate for future empirical testing of how ant

hosts cope with viral infection through illness-mediated behavioral responses, which has never been tested before simply because most previous studies have focused on fungal and/or bacterial infections (Cremer et al., 2007).

Reduced foraging activities of workers from SINV-1-infected *S. invicta* colonies also may reduce direct exposure to interspecific competition with co-existing ants, potentially increasing their survival likelihood as a result of avoidance of interference interactions with native ants. SINV-1-infected *S. invicta* colonies are significantly less competitive than their uninfected counterparts when exposed to interspecific competition with native ants (i.e., lower aggressiveness) (Chen et al., 2011). A recent field study found fire ant workers significantly outnumber workers of other native ant at the baits placed at sites with low SINV-1 prevalence, whereas fire ants account for an extremely low proportion of ants at the baits at sites with high SINV-1 prevalence (Hsu and Yang unpublished data). These field survey results, coupled with our findings, are consistent with predictions of the ‘‘resource dominance-parasitoid vulnerability trade-off theory’’ previously proposed by Feener (Feener, 2000). In this case, local coexistence of ant species within communities likely is influenced by highly specialized pathogens, such as SINV-1, that alter interspecific interactions between competing ants via changes in behaviors of dominant species such as *S. invicta*. From an applied point of view, these results further suggest the biocontrol potential of SINV-1, and increasing field prevalence of SINV-1 (e.g., artificial inoculation (Valles et al., 2013)) may practically suppress fire ants both through alterations of the foraging activities and disruption of the competitive dominance of fire ants over native ants.

Reduced foraging activity of SINV-1-infected *S. invicta* could be among one of several illness consequences associated with this viral infection. Recently, Benaets et al found that the deformed wing virus (DWV), a ssRNA virus, seemed to be responsible for

reduced foraging behaviors of honey bees (Benaets et al., 2017). Moreover, most insect-infecting ssRNA viruses (including SINV-1) replicate in the intestinal epithelium (Valles, 2012), raising the possibility that increased numbers of viral particles impair digestive capabilities of the epithelium, resulting in loss of appetite and reduced foraging activity (Kazlauskas et al., 2016). Our preliminary data are partially in line with this prediction as the number of apoptotic cells in the mid-gut of the 4th instar larvae of *S. invicta* increases with time after challenge with SINV-1 (Hsu et al., 2019). Because larval conditions in a colony can act as major drivers of worker foraging activity (Ulrich et al., 2016), the reduced foraging performance of workers in our study may be indicative of changes in larval health or status (e.g., larval mortality (Valles, 2012)), or decreased hunger levels associated with widespread SINV-1-induced apoptosis in the intestinal epithelium.

Previous studies have reported illness-induced alterations in macronutrient intake, observed as an increase in carbohydrate intake, as a process of self-medication in multiple invertebrates after being challenged with a parasite or pathogen (Graham et al., 2014; Mason et al., 2014). Despite the importance of innate immunity in prevention of subsequent infection in insects, increasing numbers of studies suggest that up-regulation of anti-viral immune systems is linked to a higher mortality due to energy costs (Mason et al., 2014). Thus, compensatory feeding may be a less costly strategy as the fitness cost can be alleviated through either enhanced tolerance of microbial infections or recouping of the resources loss as a result of combating infections (Ayres and Schneider, 2009; Shikano and Cory, 2016). Although our data do not allow us to distinguish whether altered feeding preferences towards a carbohydrate-rich diet result from self-medication or compensatory feeding, the latter may be a more plausible case scenario. Previous studies suggest that a therapeutic behavior must meet four essential

criteria, one of which predicts a decrease in fitness of an uninfected individual if engaging in a therapeutic behavior (Shikano and Cory, 2016). Carbohydrate supplementation is consistently associated with enhanced fitness at both the individual and colony levels in numerous ant species including fire ants (Grover et al., 2007; Wills et al., 2015). Also, a recent study reported multiple benefits of a high-carbohydrate diet to immunity function in the ant *Ectatomma ruidum* (Kay et al., 2014). Thus, increase of carbohydrate intake in pathogen-challenged ants may be much more prevalent than previously thought and may play a role in carbohydrate resource exploitation of ecologically dominant invasive ants.

Our results have implications for current pest management strategies of fire ants and other invasive ants. A prevailing control method of fire ants is the use of poison baits. Our data raise the interesting possibility that conventional baits may be less effective against SINV-1-infected ants as a result of changes in colony feeding patterns. First, the declining foraging activities of infected ants could lead to recruitment of fewer ants to toxic baits. Second, conventional baits may be less attractive to SINV-1-infected fire ants because of a shift in diet preferences towards carbohydrate-rich food sources. This is because current baits are impregnated with soybean oil, which serves as a phagostimulant. One predicted result of the dietary changes towards carbohydrate-rich food sources and away from food sources containing high lipid content (e.g., tuna and peanut butter) is reduced bait efficacy. In support of this prediction, a recent laboratory trial showed that SINV-1-infected *S. invicta* are significantly less susceptible to two chemicals commonly used in baits (0.73% hydramethylnon and 0.0103% fipronil) compared with non-infected ants (Tufts et al., 2014). While the underlying mechanism is unknown as no further tests were conducted, the behavioral and dietary changes associated with SINV-1 infections represent a potential avenue for future study.

Alterations in food preferences also may help explain the generally higher prevalence of SINV-1 in many introduced areas where conventional baits have been applied broadly (Yang et al., 2010), given these baits likely are more effective in eliminating uninfected colonies in the field. While our results are suggestive yet consistent with our prediction, additional studies are warranted to determine whether pathogen-induced phenotypic changes do indeed influence the efficacy of fire ant monitoring and management programs.

3.5 References

Adamo, S.A., Bartlett, A., Le, J., Spencer, N., Sullivan, K., 2010. Illness-induced anorexia may reduce trade-offs between digestion and immune function. Anim. Behav. 79, 3-10.

Allen, C., Valles, S.M., Strong, C.A., 2011. Multiple virus infections occur in individual polygyne and monogyne *Solenopsis invicta* ants. J. Invertebr. Pathol. 107, 107-111.

Ayres, J.S., Schneider, D.S., 2009. The role of anorexia in resistance and tolerance to infections in *Drosophila*. PLoS Biol. 7, e1000150.

Benaets, K., Van Geystelen, A., Cardoen, D., De Smet, L., de Graaf, D.C., Schoofs, L., Larmuseau, M.H., Brettell, L.E., Martin, S.J., Wenseleers, T., 2017. Covert deformed wing virus infections have long-term deleterious effects on honeybee foraging and survival. Proc. Biol. Sci. 284.

Bos, N., Lefevre, T., Jensen, A.B., d'Ettorre, P., 2012. Sick ants become unsociable. J. Evol. Biol. 25, 342-351.

Chen, Y.C., Kafle, L., Shih, C.J., 2011. Interspecific competition between *Solenopsis invicta* and two native ant species, *Pheidole fervens* and *Monomorium chinense*. J. Econ. Entomol. 104, 614-621.

Cremer, S., Armitage, S.A., Schmid-Hempel, P., 2007. Social immunity. Curr. Biol. 17, R693-702.

Feener, D.H.J., 2000. Is the assembly of ant communities mediated by parasitoids? Oikos 90, 79-88.

Glunn, F.J., Howard, D.F., Tschinkel, W.R., 1981. food preference in colonies of the fire ant *Solenopsis invicta*. Insect Soc. 28, 217-222.

Graham, R.I., Deacutis, J.M., Pulpitel, T., Ponton, F., Simpson, S.J., Wilson, K., 2014. Locusts increase carbohydrate consumption to protect against a fungal biopesticide. J. Insect Physiol. 69, 27-34.

Grover, C.D., Kay, A.D., Monson, J.A., Marsh, T.C., Holway, D.A., 2007. Linking nutrition and behavioural dominance: carbohydrate scarcity limits aggression and activity in Argentine ants. Proc. Biol. Sci. 274, 2951-2957.

Hafer, N., 2016. Conflicts over host manipulation between different parasites and pathogens: investigating the ecological and medical consequences. Bioessays 38, 1027-1037.

Hooper-Bui, L.M., Appel, A.G., Rust, M.K., 2002. Preference of food particle size among several urban ant species. J. Econ. Entomol. 95, 1222-1228.

Hsu, H.W., Chiu, M.C., Shih, C.J., Matsuura, K., Yang, C.C.S., 2019. Apoptosis as a primary defense mechanism in response to viral infection in invasive fire ant *Solenopsis invicta*. Virology 531, 255-259.

Kay, A.D., Bruning, A.J., van Alst, A., Abrahamson, T.T., Hughes, W.O., Kaspari, M., 2014. A carbohydrate-rich diet increases social immunity in ants. Proc. Biol. Sci. 281, 20132374.

Kazlauskas, N., Klappenbach, M., Depino, A.M., Locatelli, F.F., 2016. Sickness behavior in honey bees. Front. Physiol. 7, 261.

Manfredini, F., Lucas, C., Nicolas, M., Keller, L., Shoemaker, D., Grozinger, C.M., 2014. Molecular and social regulation of worker division of labour in fire ants. Mol. Ecol. 23, 660-672.

Manfredini, F., Shoemaker, D., Grozinger, C.M., 2016. Dynamic changes in host-virus interactions associated with colony founding and social environment in fire ant queens (*Solenopsis invicta*). Ecol. Evol. 6, 233-244.

Mason, A.P., Smilanich, A.M., Singer, M.S., 2014. Reduced consumption of protein-rich foods follows immune challenge in a polyphagous caterpillar. J. Exp. Biol. 217, 2250-2260.

Nyamukondiwa, C., Addison, P., 2014. Food preference and foraging activity of ants: recommendations for field applications of low-toxicity baits. J. Insect Sci. 14, 48.

Schmid-Hempel, P., 2017. Parasites and their social hosts. Trends Parasitol. 33, 453-462.

Shikano, I., Cory, J.S., 2016. Altered nutrient intake by baculovirus-challenged insects: self-medication or compensatory feeding? J. Invertebr. Pathol. 139, 25-33.

Simone-Finstrom, M., 2017. Social immunity and the superorganism: behavioral defenses protecting honey bee colonies from pathogens and parasites. Bee World 94, 21-29.

Singer, M.S., Mason, P.A., Smilanich, A.M., 2014. Ecological immunology mediated by diet in herbivorous insects. Integr. Comp. Biol. 54, 913-921.

Stein, M.B., Thorvilson, H.G., Johnson, J.W., 1990. Seasonal changes in bait preference by red imported fire ant, *Solenopsis invicta* (Hymenoptera, Formicidae). Fla. Entomol. 73, 117-123.

Stringer, L.D., Suckling, D.M., Baird, D., Vander Meer, R.K., Christian, S.J., Lester, P.J., 2011. Sampling efficacy for the red imported fire ant *Solenopsis invicta* (Hymenoptera: Formicidae). Environ. Entomol. 40, 1276-1284.

Tragust, S., Mitteregger, B., Barone, V., Konrad, M., Ugelvig, L.V., Cremer, S., 2013. Ants disinfect fungus-exposed brood by oral uptake and spread of their poison. Curr. Biol. 23, 76-82.

Tufts, D.M., Hunter, W.B., Bextine, B., 2014. *Solenopsis invicta* virus (SINV-1) infection and insecticide interactions in the red imported fire ant (Hymenoptera: Formicidae). Fla. Entomol. 97, 1251-1254.

Ugelvig, L.V., Cremer, S., 2007. Social prophylaxis: group interaction promotes collective immunity in ant colonies. Curr. Biol. 17, 1967-1971.

Ulrich, Y., Burns, D., Libbrecht, R., Kronauer, D.J., 2016. Ant larvae regulate worker foraging behavior and ovarian activity in a dose-dependent manner. Behav. Ecol. Sociobiol. 70, 1011-1018.

Valles, S.M., 2012. Positive-strand RNA viruses infecting the red imported fire ant, *Solenopsis invicta*. Psyche 2012, 1-14.

Valles, S.M., Hashimoto, Y., 2009. Isolation and characterization of *Solenopsis invicta* virus 3, a new positive-strand RNA virus infecting the red imported fire ant, *Solenopsis invicta*. Virology 388, 354-361.

Valles, S.M., Porter, S.D., 2003. Identification of polygyne and monogyne fire ant colonies (*Solenopsis invicta*) by multiplex PCR of *Gp-9* alleles. Insect Soc. 50, 199-200.

Valles, S.M., Porter, S.D., Choi, M.Y., Oi, D.H., 2013. Successful transmission of *Solenopsis invicta* virus 3 to *Solenopsis invicta* fire ant colonies in oil, sugar, and cricket bait formulations. J. Invertebr. Pathol. 113, 198-204.

Valles, S.M., Porter, S.D., Firth, A.E., 2014. *Solenopsis invicta* virus 3: pathogenesis and stage specificity in red imported fire ants. Virology 460, 66-71.

Valles, S.M., Strong, C.A., Dang, P.M., Hunter, W.B., Pereira, R.M., Oi, D.H., Shapiro, A.M., Williams, D.F., 2004. A picorna-like virus from the red imported fire ant, *Solenopsis invicta*: initial discovery, genome sequence, and characterization. Virology 328, 151-157.

Valles, S.M., Varone, L., Ramirez, L., Briano, J., 2009. Multiplex detection of Solenopsis invicta viruses -1, -2, and -3. J. Virol. Methods 162, 276-279.

Vander Meer, R.K., Lofgren, C.S., Seawright, J.A., 1995. Specificity of the red imported fire ant (Hymenoptera, Formicidae) phagostimulant response to carbohydrates. Fla. Entomol. 78, 144-154.

Wills, B.D., Chong, C.D., Wilder, S.M., Eubanks, M.D., Holway, D.A., Suarez, A.V., 2015. Effect of carbohydrate supplementation on investment into offspring number, size, and condition in a social insect. PLoS One 10, e0132440.

Yang, C.C., Yu, Y.C., Valles, S.M., Oi, D.H., Chen, Y.C., Shoemaker, D., Wu, W.J., Shih, C.J., 2010. Loss of microbial (pathogen) infections associated with recent invasions of the red imported fire ant *Solenopsis invicta*. Biol. Invasions 12, 3307-3318.

Zeng Y, H.X., Suh SJ., 2014. Multiple antibacterial activities of proteinaceous compounds in crude extract from the eastern subterranean termite, *Reticulitermes flavipes* Kollar (Blattodea: Isoptera: Rhinotermitidae). Adv. Res. 2, 455-461.

Chapter 4: The association between virus prevalence and intercolonial aggression levels in the yellow crazy ant, *Anoplolepis gracilipes* (Jerdon)

4.1 Introduction

A number of traits, such as shifts in social structure, inbreeding tolerance, and adaptability to human-disturbed habitats, are disproportionally presented in invasive ants (Eyer et al., 2018a; Eyer et al., 2018b; King and Tschinkel, 2016). Among these traits, supercolonies—where workers work in physically separated colonies, and exhibit limited aggression towards each other but maintain high aggression to those from other supercolonies—are believed to be associated with the ecological success of a subset of invasive ants such as the Argentine ant (*Linepithema humile* [Mayr]), the big-headed ant (*Pheidole megacephala* [Fabricius]), and the yellow crazy ant (*Anoplolepis gracilipes* [Jerdon]) (Fournier et al., 2012; Hoffmann and Hagedorn, 2014; Van Wilgenburg et al., 2010). However, the absence of visible intercolonial boundaries may also facilitate the transmission of pathogens among colonies in the same supercolony, causing supercolony-wide epidemics if no disinfection mechanisms exist or the pathogen, with limited fitness, costs to their host (Tragust et al., 2015). The "vulnerable supercolony hypothesis" was proposed by Ugelvig and Cremer (Ugelvig and Cremer, 2012) and has been empirically tested by Tragust et al. In (Tragust et al., 2015), supercolony structure was shown to be more susceptible to fungal infections due to the high frequency of intercolonial interactions in the invasive garden ant, *Lasius neglectus*. Furthermore, such a pattern is likely more profound for viral pathogens because viruses are generally more readily transmitted horizontally than other pathogen groups (Altizer et al., 2003; Dallas et al., 2018; Kavaliers and Choleris, 2018; Valles et al., 2013). Consequently, one would predict that a population comprised of mutually tolerant colonies likely has a higher level of viral prevalence as a result of intensive intercolonial interactions, while variations of prevalence should be registered in a population with mixed supercolonial groups (i.e., aggression is only observed when

workers are from different supercolonies). To assess such transmission dynamics, a critical step is to understand if intercolonial interactions serve as a potential transmission pathway of a given virus.

Native to the Paleotropics (but see (Wetterer, 2005)), the yellow crazy ant, *A. gracilipes,* was introduced into many islands in the Indian and Pacific Oceans and had destructive impacts on the native ecosystems on these islands (Abbott, 2005; Green et al., 2011). Despite the presence of supercolonies in most of these island populations, previous studies have shown that populations of *A. gracilipes* in Christmas Island (Abbott, 2005) and Taiwan (Lee, unpublished data) generally comprise of colonies that display limited aggression toward each other, while those in Penang, Malaysia, harbor a high proportion of mutually aggressive colonies (Chong and Lee, 2010). These behavioral observations suggest that population structure may vary across where this ant is distributed. Coupled with the recent discovery of the TR44839 virus in the yellow crazy ant (Cooling et al., 2017), such differences in intercolonial aggression behavior provide an excellent opportunity to test whether viral prevalence is associated with the social interactions of populations. The TR44839 virus was discovered in a confirmed invasive population of *A. gracilipes* in Australia and appears to be a member of the *Dicistroviridae* viral family based on phylogenetic analysis (Cooling et al., 2017). Despite no detailed fundamental information reported from the same study (Cooling et al., 2017), viruses in this family are generally characterized as positive-sense, single-stranded RNA genomes, and usually persist in hosts asymptomatically, yet sometimes cause illness in their arthropod hosts (Bonning, 2009).

Colony organization and interaction network play a critical role in regulating disease transmission dynamics in social insects. A majority of studies, however, focused on an intracolony level, and the potential effects from higher hierarchical levels (e.g.,

intercolony), are rarely tested (but see (Tragust et al., 2015)). In this study, we hypothesized that viral prevalence is lower (or more variable) in populations of *A. gracilipes* characterized primarily by mutually aggressive colonies than those with colonies displaying none or limited aggression level. If such an association holds, the aggression between colonies is likely correlated with the viral prevalence. We first mapped the viral distribution among the colonies of *A. gracilipes* in numerous sites on three Asian islands (Okinawa, Japan; Taiwan; Penang, Malaysia) and assayed aggressive interactions between colonies at both within-site and between-site levels. We then examined if a correlation exists between the two variables in *A. gracilipes*. We believe that this is the first study that dissects the interplay between social interactions and viral prevalence in *A. gracilipes*, and offers critical information that would assist in developing an efficient virus-based pest management strategy.

4.2 Materials and methods

Virus detection and confirmation of viral replication

We selected a total of 12 sites from three islands of varying latitudes, namely Okinawa, Japan (N = 2, JP1 & 2), Taiwan (N = 7, TW1-7) and Penang, Malaysia (N = 3, MY1-3), and collected various numbers of *A. gracilipes* colonies in each site (Figure 4.1). The colony in the current study was defined as a physical nest unit, containing all castes/stages (queens, workers and brood, and sometimes alates), clearly delineated from another unit by at least 100 m. A total of 28, 27 and 40 colonies (all sites combined) were collected from Okinawa, Taiwan, and Penang, respectively. We screened for the presence of the TR44839 virus in each site and calculated the infection rate based on the number of infected colonies over the total number of collected colonies in a site. To understand if the TR44839 virus can be horizontally transmitted among colonies, and also if the virus actively replicates in *A. gracilipes*, an artificial inoculation assay of the

virus was carried out. Additionally, three colonies confirmed to be TR44839 virus-uninfected (note, these three colonies were not involved in subsequent aggression assay) were collected and fed with a fixed amount of 10% honey solution homogenized with virus-infected *A. gracilipes* workers for 24 h. Given the fact that the replication of the virus in the *Dicistroviridae* family proceeds via the synthesis of a complementary negative strand, the presence of a negative strand would serve as evidence of replication. We, therefore, attempted to detect the negative strand of the virus using strand-specific RT-PCR at Day 7 after feeding. The RNA of five workers from each colony was extracted and used as the template for cDNA synthesis, using oligo-dT primer and the strand-specific primer. The resultant cDNA was then added to the subsequent PCR reaction in which the target viral fragment was amplified using the previously reported primer set (TR44839: AGACGAACGAACAAAGGACCT / CAATTCAGTTCTGCGGGTGC) (Cooling et al., 2017). The PCR amplification protocol included 94 °C for 3 min, then 35 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension step of 72 °C for 5 min. Samples were confirmed TR44839-virus-positive if a 130 bp fragment was visualized on the agarose gel electrophoresis. We included a positive control (RNA extracted from individuals previously confirmed positive by both gel electrophoresis and sequencing), negative control (RNA extracted from individuals previously confirmed negative for the virus), and blank (ddH_2O) in every batch of RT-PCR reaction.

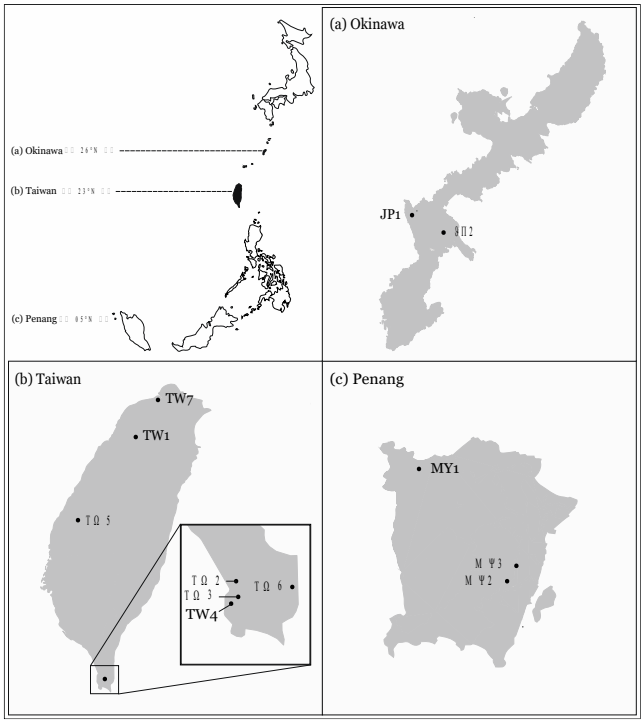

Figure 4.1 Geographic distribution of our study sites in (a) Okinawa (JP1 and JP2), (b) Taiwan (TW1-TW7), and (c) Penang (MY1-MY3).

Aggression assay

To assess the pattern of within-site intercolonial aggression in *A. gracilipes*, we randomly selected three colonies from each site and assayed aggression between workers from different colonies within the same site, resulting in 6, 21 and 9 pairwise aggression tests in Okinawa, Taiwan and Penang, respectively (Figure 4.2a; Appendix 4). The aggression assay was carried out within a week of the field collection. An individual worker sampled from each of two colonies was placed in a 6 cm diameter petri dish with fluon-coated inner wall surface for 5 min. Each individual was tested only once and discarded. The behavioral interactions between two workers during a 5 min period were observed and scored as follows: 1 for ignoring or touching (antennation); 2 for avoidance; 3 for aggression (lunging, biting, or pulling); 4 for fighting (prolonged aggression) (Suarez et al., 1999). Ten replicates (ten individual workers) were performed for each colony pair, and aggression levels were averaged

(Appendix 4). Aggression assays were scored when the maximum level of aggression was achieved by two workers during each trial. For example, the observation was terminated if level 4 had been achieved before the end of the 5 min period. To understand the differences in overall intercolonial aggression levels between the three islands, aggression levels derived from all colony pairs, involving two colonies collected from the same island, were pooled and their significance analyzed using the Kruskal–Wallis rank-sum test, and the pairwise comparison analyzed using the Wilcoxon rank-sum test with Bonferroni correction.

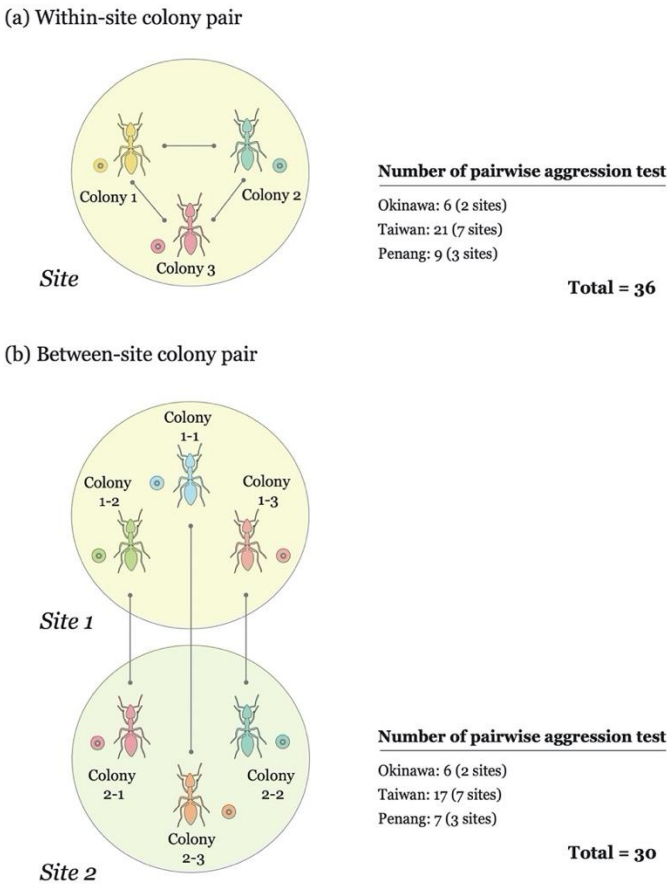

Figure 4.2 Graphic summary for within-site (a) and between-site (b) aggression assays. Colony pairs in the within-site aggression assay involve two colonies collected from the same site, while colony pairs involved in the between-site aggression assay are those collected from different sites of the same island. The number of pairwise aggression tests is indicated.

Correlation between infection status and aggressive behavior

Intercolonial relationships (e.g., colony interactions) generally play an important role in shaping the efficiency of disease transmissions in social insects (Berville et al., 2013; Naug and Camazine, 2002). For example, high aggression between individual workers from different colonies is believed to decrease pathogen transmissions due to the limited contact and/or high causality and mortality following hostile interactions (Konrad et al., 2018). We, therefore, aimed to test the pathogen infection patterns associated with intercolonial aggression by analyzing the correlation between infection status and aggressive level. Additional aggression assay was carried out on colony pairs involving colonies from different sites of the same island (hereafter referred to as between-site colony pair, n = 30; Figure 4.2b; Appendix 4), and the data were combined with that of the within-site colony pairs to ensure the dataset is statistically robust. Not all between-site colony pairs were subjected to the aggression test, particularly those from Taiwan, due to different field collection schemes. We then categorized all the colony pairs into three groups, based on the infection status of the two colonies involved, those groups being uninfected vs. uninfected (−/−), infected vs. uninfected (−/+), and infected vs. infected (+/+). In addition, the Kruskal–Wallis rank-sum test, and the pairwise comparisons using the Wilcoxon rank-sum test with Bonferroni correction, were used to determine whether aggression levels are associated with the infection status. We conducted the non-parametric hypothesis tests and data visualization with the basic function, fitted the linear mixed model using the lme4 package, and selected the best fitted model by MuMIn package in *R* software (version 3.5.2, *R* Core Team, 2018).

4.3 Results

We inoculated three virus-free *A. gracilipes* colonies by feeding them with a 10% virus-contaminated honey solution, and all the colonies were invariably found positive to TR44839 virus infection as both positive and negative strands of the virus were detected at Day 7 after feeding (Appendix 5). Hence, we confirmed that the TR44839 virus can be horizontally transmitted among colonies and actively replicate in *A. gracilipes*. TR44839 virus occurred in all *A. gracilipes* colonies in Okinawa and Taiwan (infection rate 100%), while variations in viral prevalence were observed across the study sites in Penang. Virus-infected colonies in Penang were mostly found in MY1 (100%), whereas the infection rate was lower in sites MY2 (31%), and none in MY3 (0%) (Figure 4.3).

We found significant differences in aggression levels between the three islands (Kruskal–Wallis rank-sum test: $\chi^2 = 21.087$ df = 2, *p*-Value < 0.001) (Appendix 6). In general, colonies collected from Okinawa were characterized by the lowest within-site aggression level. Interestingly, individual workers from within-site colony pairs in Taiwan frequently displayed "biting", but rarely "prolonged aggression". The highest level of within-site aggression (i.e., level 4) was commonly observed in colony pairs involving colonies collected from the Penang island (Figure 4.3). Site differences in aggression behavior were observed in the colonies in Taiwan and Penang (Figure 4.3). In Taiwan, the within-site aggression level could be divided into two groups, with one group showing little aggression (TW1-TW4) and the other group showing high aggression (TW5-TW7). Mapping the aggression level onto the map revealed a general trend in which aggressive behavior decreased as the latitude increased (Figure 4.3; Appendix 6). Moreover, the colonies collected from sites in MY2 and MY3 displayed a high level of aggression, even though the colonies were in close geographic proximity.

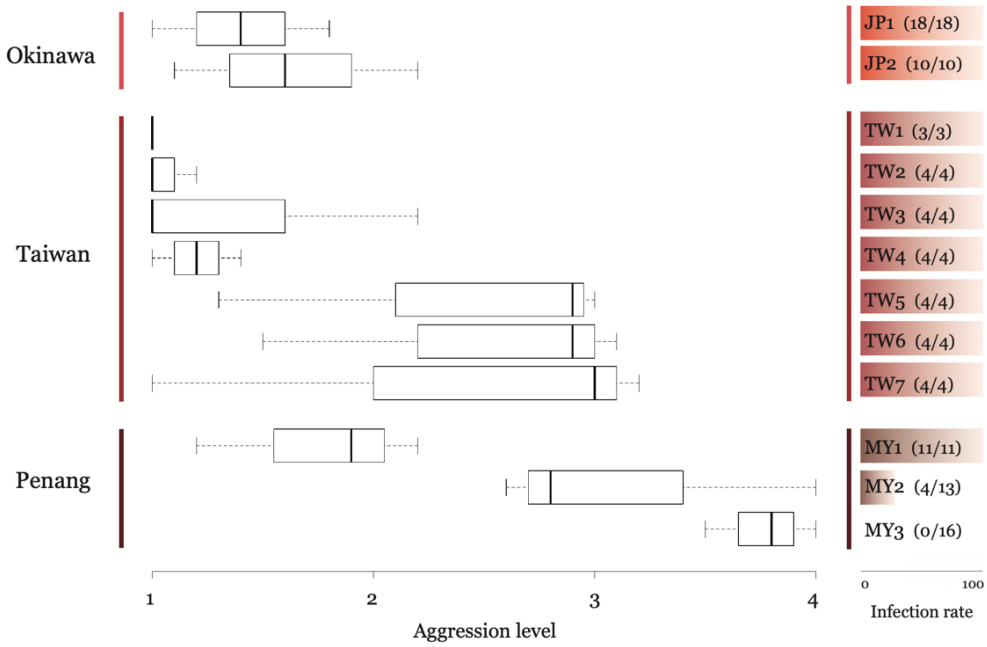

Figure 4.3 Overview of aggression level and infection rate across the study sites. Numbers in parenthesis denote the number of infected colonies/number of collected colonies in a given site.

Our analysis showed that aggression levels significantly differed between colony pairs with different infection statuses (Kruskal–Wallis rank-sum test: $\chi^2 = 25.63$, df = 2, *p*-Value < 0.001). For example, pairs displaying a high level of aggression generally involved one worker that was infection free, while the aggression level significantly decreased when both workers were infected (Figure 4.4).

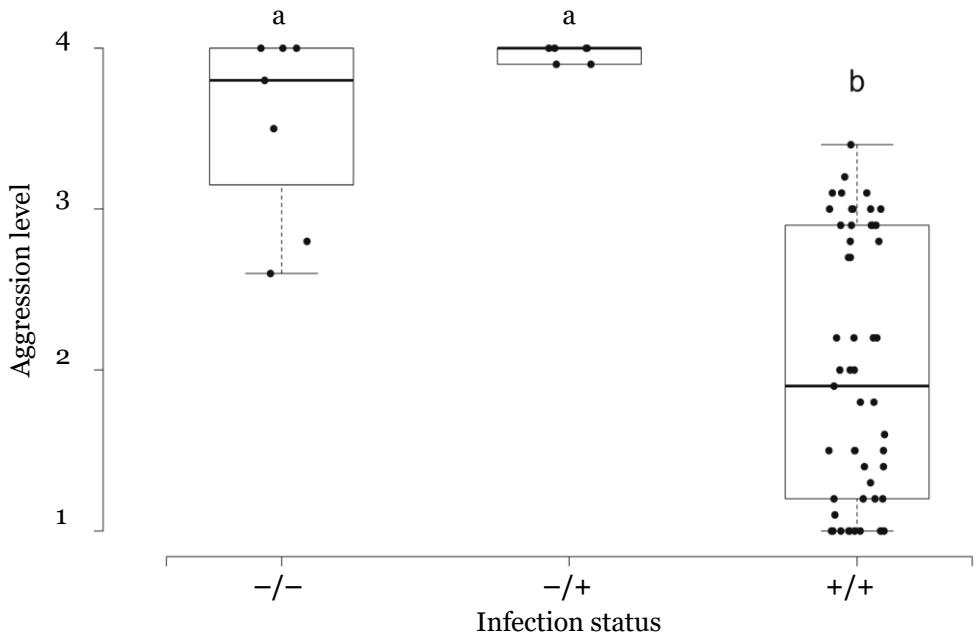

Figure 4.4 The aggression level of each colony pair in the three categories based on the infection status of interacting workers. (−/−) denotes the pair involving both workers from uninfected colonies; (−/+) denotes the pair involving one infected worker, and one uninfected worker; (+/+) denotes the pair involving both workers from infected colonies. The letters indicate the significant differences between group (p-Value < 0.001).

4.4 Discussion

This study reported that the TR44839 virus is widespread in most study sites, particularly in those sites with *A. gracilipes* displaying low to moderate intercolonial aggression (i.e., Okinawa and Taiwan), but much less prevalent in sites with ants showing a high level of intercolonial aggression (i.e., Penang). A plausible explanation for the widespread nature of the TR44839 virus is that intensive interactions between colonies may have facilitated the spread of pathogens. The observed pattern in this study is analogous with the pathogen transmission dynamics at the intracolonial level, where higher frequencies of nestmate interactions can readily promote the spread of pathogens within the colony, implying that colony boundaries and levels of

intercolonial interactions may contribute to facilitating/preventing viral transmission in *A. gracilipes*.

Formation of supercolonies and the reduction of intercolonial aggression were commonly found in introduced populations of several invasive ant species including *A. gracilipes* (Abbott, 2005; Eyer et al., 2018b; Giraud et al., 2002; Krapf et al., 2018), suggesting that such alterations in social structure may be associated with invasiveness of these ant species. However, the vulnerable supercolony hypothesis predicts that recurring intercolonial contact readily offers more opportunities for pathogen transmission than species with colonies displaying a territorial boundary. Our study adds to evidence supporting the hypothesis, as we found a strong link between weak social boundaries (simple colony composition) and epidemics of the TR44839 virus among the colonies in Okinawa and Taiwan. Additional support for pathogen transmission dynamics at population level can be obtained from red imported fire ant. Polygynous fire ant colonies harbor a significantly higher diversity of parasites and pathogens when compared to their monogyne conspecifics, and such differences are attributed to the presence of intensive intercolonial interactions among polygyne colonies, such as resource and worker exchanges between colonies (Hoffmann and Hagedorn, 2014; Oi, 2006; Valles et al., 2010). Intensive intercolonial interaction as a result of social structure alteration following invasion may also help explain the finding of viruses (if present) generally persisting in a higher prevalence in introduced areas than those in the native ranges (Sébastien et al., 2015; Yang et al., 2010).

A previous study has shown that *A. gracilipes* colonies in Penang displayed a high level of intercolonial aggression, and that high mortality was common during the intraspecific aggression assay (Chong and Lee, 2010). Our findings are consistent with this earlier study, and allow us to presume that the population structure of *A. gracilipes*

in this region is heterogeneous and may harbor multiple localized supercolonies. Interestingly, we found that the intercolonial aggression level appears to decrease as the latitude increases (Figure 4.3; Appendix 6), suggesting the presence of a latitudinal gradient of aggression level and possibly population heterogeneity as well. While the origin of *A. gracilipes* remains open to debate, our data seem to favor a Southeast Asian origin for *A. gracilipes*, because the supercolony structure of this ant in Penang, Malaysia resembles that in native Argentine ants, whose supercolony is generally more localized than populations in their introduced ranges (Giraud et al., 2002; Vogel et al., 2009). Indeed, emerging evidence has been accumulated to support this ant originating from Southeast Asia. For example, an earlier study by Ito et al. (Ito et al., 2016) found several incipient colonies of *A. gracilipes* that have apparently been initiated by single solitary queens independently in East Java, Indonesia, and the discovery of *A. gracilipes* queens adopting such **"**ancestral**"** founding strategy may, therefore, imply that this area and possibly other parts of Southeast Asia could be their native range. Large-scale surveys and the development of high-resolution genetic markers are urgently warranted.

4.5 Conclusions

Numerous positive-sense, single-strain RNA viruses have been reported in multiple invasive ant species (Cooling et al., 2017; Valles, 2012; Viljakainen et al., 2018) with a wide range of effects on their host ants spanning from the reduction of foraging activity (Hsu et al., 2018) and fitness costs (Manfredini et al., 2016), to colony mortality (Valles and Hashimoto, 2009; Valles et al., 2004), allowing the utilization of these viruses as biocontrol agents to become feasible and promising (Oi et al., 2019). One necessary piece of information to evaluate the biocontrol potential of viruses lies in how these viruses are transmitted between colonies, yet most of the mechanism studies are largely based on a single ant system (i.e., red imported fire ant) and its viruses (Chen et

al., 2006; Oi et al., 2019). While other factors may have been involved, the current study has demonstrated that aggression levels among colonies may represent one contributor in shaping viral disease transmission in *A. gracilipes*. Future study should examine if intercolonial aggression remains an effective factor associated with virus transmission in other ant-virus systems, as well as the role of other biotic and abiotic factors.

4.6 References

Abbott, K.L., 2005. Supercolonies of the invasive yellow crazy ant, *Anoplolepis gracilipes*, on an oceanic island: forager activity patterns, density and biomass. Insect Soc. 52, 266-273.

Altizer, S., Nunn, C.L., Thrall, P.H., Gittleman, J.L., Antonovics, J., Cunningham, A.A., Dobson, A.P., Ezenwa, V., Jones, K.E., Pedersen, A.B., Poss, M., Pulliam, J.R.C., 2003. Social organization and parasite risk in mammals: integrating theory and empirical studies. Annu. Rev. Ecol. Evol. Syst. 34, 517-547.

Berville, L., Blight, O., Renucci, M., Hefetz, A., Provost, E., 2013. A peaceful zone bordering two Argentine ant (*Linepithema humile*) supercolonies. Chemoecology 23, 213-218.

Bonning, B.C., 2009. The Dicistroviridae: an emerging family of invertebrate viruses. Virol. Sin. 24, 415-427.

Chen, Y., Evans, J., Feldlaufer, M., 2006. Horizontal and vertical transmission of viruses in the honey bee, *Apis mellifera*. J. Invertebr. Pathol. 92, 152-159.

Chong, K.F., Lee, C.Y., 2010. Inter- and intraspecific aggression in the invasive longlegged ant (Hymenoptera: Formicidae). J. Econ. Entomol. 103, 1775-1783.

Cooling, M., Gruber, M.A.M., Hoffmann, B.D., Sebastien, A., Lester, P.J., 2017. A metatranscriptomic survey of the invasive yellow crazy ant, *Anoplolepis*

gracilipes, identifies several potential viral and bacterial pathogens and mutualists. Insect Soc. 64, 197-207.

Dallas, T.A., Krkosek, M., Drake, J.M., 2018. Experimental evidence of a pathogen invasion threshold. R. Soc. Open Sci. 5, 171975.

Eyer, P.A., Matsuura, K., Vargo, E.L., Kobayashi, K., Yashiro, T., Suehiro, W., Himuro, C., Yokoi, T., Guenard, B., Dunn, R.R., Tsuji, K., 2018a. Inbreeding tolerance as a pre-adapted trait for invasion success in the invasive ant *Brachyponera chinensis*. Mol. Ecol. 27, 4711-4724.

Eyer, P.A., McDowell, B., Johnson, L.N.L., Calcaterra, L.A., Fernandez, M.B., Shoemaker, D., Puckett, R.T., Vargo, E.L., 2018b. Supercolonial structure of invasive populations of the tawny crazy ant *Nylanderia fulva* in the US. BMC Evol. Biol. 18, 209.

Fournier, D., Tindo, M., Kenne, M., Mbenoun Masse, P.S., Van Bossche, V., De Coninck, E., Aron, S., 2012. Genetic structure, nestmate recognition and behaviour of two cryptic species of the invasive big-headed ant *Pheidole megacephala*. PLoS One 7, e31480.

Giraud, T., Pedersen, J.S., Keller, L., 2002. Evolution of supercolonies: the Argentine ants of southern Europe. Proc. Natl. Acad. Sci. U. S. A. 99, 6075-6079.

Green, P.T., O'Dowd, D.J., Abbott, K.L., Jeffery, M., Retallick, K., Mac Nally, R., 2011. Invasional meltdown: invader-invader mutualism facilitates a secondary invasion. Ecology 92, 1758-1768.

Hoffmann, B.D., Hagedorn, H., 2014. Quantification of supercolonial traits in the yellow crazy ant, *Anoplolepis gracilipes*. J. Insect Sci. 14, 25.

Hsu, H.W., Chiu, M.C., Shoemaker, D., Yang, C.S., 2018. Viral infections in fire ants lead to reduced foraging activity and dietary changes. Sci. Rep. 8, 13498.

Ito, F., Asfiya, W., Kojima, J., 2016. Discovery of independent-founding solitary queens in the yellow crazy ant *Anoplolepis gracilipes* in East Java, Indonesia (Hymenoptera: Formicidae). Entomol. Sci. 19, 312-314.

Kavaliers, M., Choleris, E., 2018. The role of social cognition in parasite and pathogen avoidance. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 373, 20170206.

King, J.R., Tschinkel, W.R., 2016. Experimental evidence that dispersal drives ant community assembly in human-altered ecosystems. Ecology 97, 236-249.

Konrad, M., Pull, C.D., Metzler, S., Seif, K., Naderlinger, E., Grasse, A.V., Cremer, S., 2018. Ants avoid superinfections by performing risk-adjusted sanitary care. Proc. Natl. Acad. Sci. U. S. A. 115, 2782-2787.

Krapf, P., Russo, L., Arthofer, W., Most, M., Steiner, F.M., Schlick-Steiner, B.C., 2018. An Alpine ant's behavioural polymorphism: monogyny with and without internest aggression in *Tetramorium alpestre*. Ethol. Ecol. Evol. 30, 220-234.

Manfredini, F., Shoemaker, D., Grozinger, C.M., 2016. Dynamic changes in host-virus interactions associated with colony founding and social environment in fire ant queens (*Solenopsis invicta*). Ecol. Evol. 6, 233-244.

Naug, D., Camazine, S., 2002. The role of colony organization on pathogen transmission in social insects. J. Theor. Biol. 215, 427-439.

Oi, D., Valles, S., Porter, S., Cavanaugh, C., White, G., Henke, J., 2019. Introduction of fire ant biological control agents into the Coachella Valley of California. Fla. Entomol. 102, 284-286.

Oi, D.H., 2006. Effect of mono- and polygyne social forms on transmission and spread of a microsporidium in fire ant populations. J. Invertebr. Pathol. 92, 146-151.

Sébastien, A., Lester, P.J., Hall, R.J., Wang, J., Moore, N.E., Gruber, M.A.M., 2015. Invasive ants carry novel viruses in their new range and form reservoirs for a honeybee pathogen. Biol. Lett. 11, 20150610.

Suarez, A.V., Tsutsui, N.D., Holway, D.A., Case, T.J., 1999. Behavioral and genetic differentiation between native and introduced populations of the Argentine ant. Biol. Invasions 1, 43-53.

Tragust, S., Feldhaar, H., Espadaler, X., Pedersen, J.S., 2015. Rapid increase of the parasitic fungus Laboulbenia formicarum in supercolonies of the invasive garden ant Lasius neglectus. Biological Invasions 17, 2795-2801.

Ugelvig, L.V., Cremer, S., 2012. Effects of social immunity and unicoloniality on host-parasite interactions in invasive insect societies. Funct. Ecol. 26, 1300-1312.

Valles, S.M., 2012. Positive-strand RNA viruses infecting the red imported fire ant, *Solenopsis invicta*. Psyche 2012, 1-14.

Valles, S.M., Hashimoto, Y., 2009. Isolation and characterization of *Solenopsis invicta* virus 3, a new positive-strand RNA virus infecting the red imported fire ant, *Solenopsis invicta*. Virology 388, 354-361.

Valles, S.M., Oi, D.H., Porter, S.D., 2010. Seasonal variation and the co-occurrence of four pathogens and a group of parasites among monogyne and polygyne fire ant colonies. Biol. Control 54, 342-348.

Valles, S.M., Porter, S.D., Choi, M.Y., Oi, D.H., 2013. Successful transmission of *Solenopsis invicta* virus 3 to *Solenopsis invicta* fire ant colonies in oil, sugar, and cricket bait formulations. J. Invertebr. Pathol. 113, 198-204.

Valles, S.M., Strong, C.A., Dang, P.M., Hunter, W.B., Pereira, R.M., Oi, D.H., Shapiro, A.M., Williams, D.F., 2004. A picorna-like virus from the red imported fire ant,

Solenopsis invicta: initial discovery, genome sequence, and characterization. Virology 328, 151-157.

Van Wilgenburg, E., Torres, C.W., Tsutsui, N.D., 2010. The global expansion of a single ant supercolony. Evol. Appl. 3, 136-143.

Viljakainen, L., Holmberg, I., Abril, S., Jurvansuu, J., 2018. Viruses of invasive Argentine ants from the European Main supercolony: characterization, interactions and evolution. J. Gen. Virol. 99, 1129-1140.

Vogel, V., Pedersen, J.S., d'Ettorre, P., Lehmann, L., Keller, L., 2009. Dynamics and genetic structure of Argentine ant supercolonies in their native range. Evolution 63, 1627-1639.

Wetterer, J.K., 2005. World-wide distribution and potential spread of the long-legged ant, *Anoplolepis gracilipes* (Hymenoptera: Formicidae). Sociobiology 45, 77-97.

Yang, C.C., Yu, Y.C., Valles, S.M., Oi, D.H., Chen, Y.C., Shoemaker, D., Wu, W.J., Shih, C.J., 2010. Loss of microbial (pathogen) infections associated with recent invasions of the red imported fire ant *Solenopsis invicta*. Biol. Invasions 12, 3307-3318.

Chapter 5: Conclusions

Bait broadcast has been an efficient approach to control invasive ants, while little effort was directed to integrated management of invasive ants. Recently, a few viruses have been found in numerous ant species in their introduced populations, and such findings offer an alternative strategy in controlling these widespread invaders. This thesis consists of three main parts. The first part is devoted to characterizing host-pathogen dynamics of the red imported fire ant (*Solenopsis invicta*) and *Solenopsis invicta* virus 1 (SINV-1). Previous studies showed that SINV-1 exhibits tissue tropism to midgut tissue of fire ant larvae, raising the possibility of potential interactions between midgut and the virus. We showed that viral titer is negatively correlated with apoptosis level in the midgut epithelium, a pattern consistent with the fact that apoptosis in the midgut epithelium is a primary cellular defense mechanism in insects to viral infection. Given that late-instar larva of *S. invicta* are the primary colony members that digest solid food and also redistribute the food through trophallaxis in liquid form to nestmates, we hypothesized that such apoptosis in the midgut of late-instar larvae may result into alterations in foraging behavior at colony level. The second part of the thesis thus examined if the virus infection leads to changes in foraging activity and food preference in *S. invicta*. Our results showed that infected colony fragments consistently displayed reduced foraging activity and a shift in dietary preference to carbohydrate-rich foods compared with virus-free fragments. Combined with additional observations, such behavioral alterations may represent a possible mechanism underlying how fire ant colonies respond to viral epidemics. Although utilization of ant viruses as a biocontrol agent is considered feasible and promising, one necessary piece of information before field release of viral pathogen lies in understanding of their transmission dynamics among colonies. The final part of the thesis is to characterize the association between

field prevalence of a virus and intercolonial aggression of its ant host, using the yellow crazy ant (*Anoplolepis gracilipes*) and its virus (TR44839 virus) as a model system. Our results indicated the viral prevalence is lower in *A. gracilipes* populations characterized primarily by mutually aggressive colonies than those with colonies displaying none or limited aggression level, suggesting interaction network among colonies may represent one contributor in shaping viral prevalence in the field. Potential pest management implications on invasive ants using viral pathogens are also discussed.

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Appendix 1

Appendix 1 Colony fragment profiles (geographic information, queen and infection status)

Colony code	Collection site	Queen located	
		uninfected	Infected
Si-308	New Taipei City		√
Si-309	New Taipei City	√	
Si-310	New Taipei City		√
Si-304	Taoyuan City		√
Si-336	Taoyuan City		√
Si-339	Taoyuan City	√	
Si-331	New Taipei City	√	
Si-342	Taoyuan city		√
Si-326	New Taipei City	√	

Appendix 2

Appendix 2 Macronutrient composition of the four food resources for food preference test (%)

	Carbohydrate	Protein	Lipid	Others (minor)
Honey	82.4	0.3	0	17.3
Potato chip	59.7	4.8	30.5	5
Tuna	14.5	36.2	40	9.3
Peanut butter	20	25	50	5

Appendix 3

Appendix 3 The pairwise comparisons of foraging intensity, time required to foraging onset and recruitment efficiency

	Uninfected w/ queen	Uninfected w/o queen	Infected w/ queen	Infected w/o queen
Foraging intensity (n)	14778.75±4894.11[a]	14004.80±10813.80[a]	921.40±446.12[b]	613.25±417.10[b]
Onset of foraging (min)[1]	17.50±9.57	18.00±13.04	310.00±504.58	135.00±113.58
Recruitment efficiency (min)	85.00±36.97[a]	92.00±44.94[a]	622.00±501.47[b]	1452.50±169.39[c]

[1] The pairwise comparison was not preformed among the time required to foraging onset since the differences is not significant in Kruskal-Wallis test.

[a–d] Letters indicate significant pairwise differences (multiple comparisons conducted using a Tukey's honestly significant difference test (foraging intensity) and pairwise T test (recruitment efficiency), $P < 0.05$) across the various categories for each segment.

Appendix 4

Appendix 4 Results for within-site and between-site aggression tests

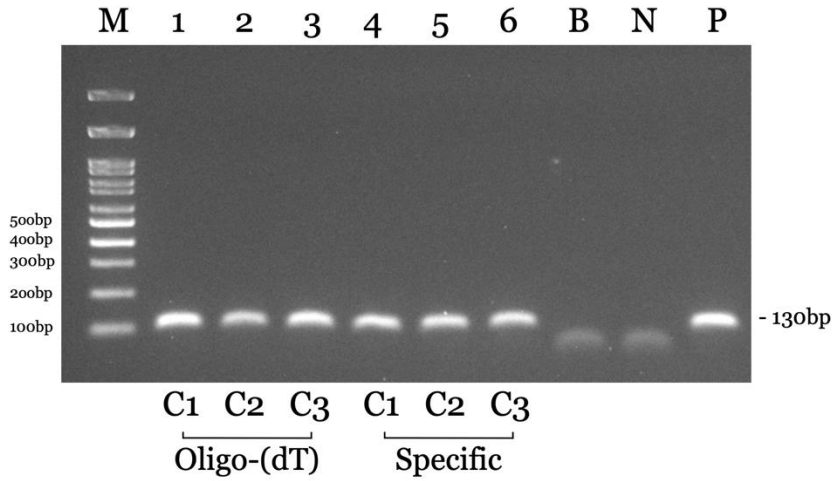
Whitin-site colony pair

Region	Site	Pair	Distance (Km)	Infection status	Aggression assay (10 replicates)										
					1	2	3	4	5	6	7	8	9	10	Average
Okinawa	1	1-1 vs 1-3	0.152	(+/+)	2	2	1	1	3	3	2	2	1	1	1.8
Okinawa	1	1-2 vs 1-3	0.167	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Okinawa	1	1-1 vs 1-2	0.117	(+/+)	3	1	1	3	1	1	1	1	1	1	1.4
Okinawa	2	2-1 vs 2-3	0.149	(+/+)	1	1	1	1	1	1	1	2	1	1	1.1
Okinawa	2	2-2 vs 2-3	0.12	(+/+)	2	1	2	1	2	3	1	1	2	1	1.6
Okinawa	2	2-1 vs 2-2	0.121	(+/+)	2	1	1	1	3	2	3	3	3	3	2.2
Taiwan	1	1-1 vs 1-3	0.104	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	1	1-2 vs 1-3	0.103	(+/+)	2	1	1	1	2	1	1	1	1	1	1.2
Taiwan	1	1-1 vs 1-2	0.16	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	2	2-1 vs 2-3	0.18	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	2	2-2 vs 2-3	0.123	(+/+)	1	3	3	3	3	1	3	1	1	3	2.2
Taiwan	2	2-1 vs 2-2	0.128	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	3	3-1 vs 3-3	0.149	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	3	3-2 vs 3-3	0.133	(+/+)	1	1	2	1	2	3	1	1	1	1	1.4
Taiwan	3	3-1 vs 3-2	0.11	(+/+)	1	1	2	1	1	2	1	1	1	1	1.2
Taiwan	4	4-1 vs 4-3	0.15	(+/+)	3	3	3	3	3	3	3	3	3	3	3
Taiwan	4	4-2 vs 4-3	0.29	(+/+)	4	3	3	3	3	4	3	3	3	3	3.2
Taiwan	4	4-1 vs 4-2	0.444	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	5	5-1 vs 5-3	0.528	(+/+)	1	1	1	1	1	1	3	1	1	2	1.3
Taiwan	5	5-2 vs 5-3	0.42	(+/+)	3	3	3	3	3	3	3	3	3	3	3
Taiwan	5	5-1 vs 5-2	0.273	(+/+)	3	3	3	3	3	3	2	3	3	3	2.9
Taiwan	6	6-1 vs 6-3	2.32	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	6	6-2 vs 6-3	3.69	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	6	6-1 vs 6-2	1.74	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	7	7-1 vs 7-3	7.61	(+/+)	3	3	3	3	3	3	3	3	2	3	2.9
Taiwan	7	7-2 vs 7-3	6.48	(+/+)	3	3	3	3	3	3	3	4	3	3	3.1
Taiwan	7	7-1 vs 7-2	1.78	(+/+)	1	1	1	2	1	3	1	3	1	1	1.5
Penang	1	1-1 vs 1-3	0.606	(+/−)	4	4	4	4	4	4	4	4	4	4	4
Penang	1	1-2 vs 1-3	0.579	(−/−)	4	3	3	4	4	3	4	3	3	4	3.5
Penang	1	1-1 vs 1-2	0.157	(−/−)	4	4	4	4	3	4	4	3	4	4	3.8
Penang	2	2-1 vs 2-3	1.25	(−/−)	3	3	3	2	3	2	3	3	3	3	2.8
Penang	2	2-2 vs 2-3	0.156	(−/−)	3	3	3	2	3	3	1	2	3	3	2.6
Penang	2	2-1 vs 2-2	1.35	(+/−)	4	4	4	4	4	4	4	4	4	4	4
Penang	3	3-1 vs 3-3	0.227	(+/+)	3	3	3	3	1	1	1	1	3	3	2.2
Penang	3	3-2 vs 3-3	0.12	(+/+)	1	1	2	1	3	3	3	3	1	1	1.9
Penang	3	3-1 vs 3-2	0.256	(+/+)	1	1	1	1	3	1	1	1	1	1	1.2

Between-site colony pair

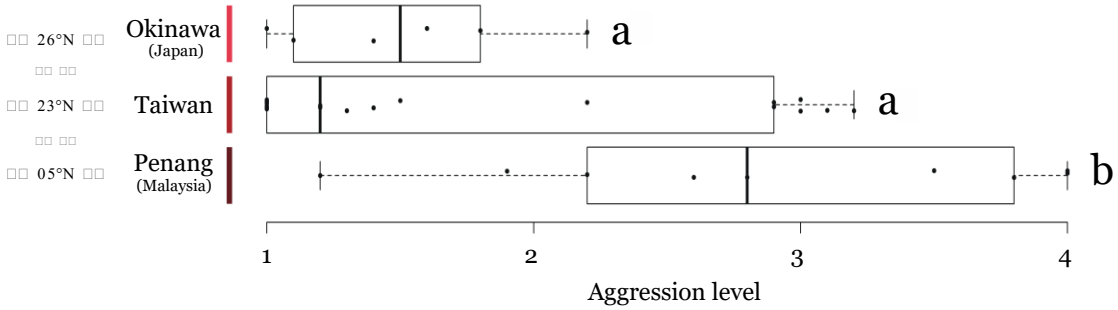
Region	Site	Pair	Distance (Km)	Infection status	Aggression assay (10 replicates)										
					1	2	3	4	5	6	7	8	9	10	Average
Okinawa	1 vs 2	1-3 vs 2-3	11.5	(+/+)	1	1	2	1	3	3	3	3	2	1	2
Okinawa	1 vs 2	1-1 vs 2-3	12.3	(+/+)	1	1	2	2	3	1	1	1	2	1	1.5
Okinawa	1 vs 2	1-3 vs 2-2	11.7	(+/+)	2	3	3	1	1	1	1	1	1	1	1.5
Okinawa	1 vs 2	1-3 vs 2-1	11.5	(+/+)	1	1	1	1	3	3	3	3	1	1	1.8
Okinawa	1 vs 2	1-2 vs 2-2	11.4	(+/+)	1	1	1	1	3	3	2	1	1	1	1.5
Okinawa	1 vs 2	1-2 vs 2-3	11.2	(+/+)	3	1	1	1	1	1	1	1	1	1	1.2
Taiwan	1 vs 2	1-1 vs 2-3	0.531	(+/+)	3	3	3	3	3	3	3	3	4	3	3.1
Taiwan	1 vs 2	1-1 vs 2-1	0.527	(+/+)	3	3	3	3	3	3	1	3	3	3	2.8
Taiwan	1 vs 2	1-3 vs 2-3	0.47	(+/+)	3	3	3	2	3	3	3	3	3	3	2.9
Taiwan	1 vs 2	1-1 vs 2-2	0.49	(+/+)	3	3	4	3	3	3	1	3	1	3	2.7
Taiwan	1 vs 3	1-1 vs 3-1	0.168	(+/+)	1	1	1	1	1	1	1	1	1	1	1
Taiwan	1 vs 3	1-3 vs 3-3	0.126	(+/+)	1	3	3	3	3	1	1	1	1	3	2
Taiwan	1 vs 5	1-1 vs 5-2	177	(+/+)	3	3	3	3	2	3	2	4	3	4	3
Taiwan	1 vs 5	1-1 vs 5-3	177	(+/+)	3	3	3	3	3	3	3	3	4	3	3.1
Taiwan	2 vs 3	2-2 vs 3-3	0.543	(+/+)	3	1	3	3	4	3	1	4	3	4	2.9
Taiwan	2 vs 3	2-3 vs 3-3	0.528	(+/+)	4	3	3	3	3	1	3	3	3	3	2.9
Taiwan	2 vs 4	2-1 vs 4-3	7.45	(+/+)	3	3	3	3	3	3	3	1	3	3	2.8
Taiwan	2 vs 4	2-2 vs 4-3	7.44	(+/+)	1	3	3	3	3	1	1	3	1	3	2.2
Taiwan	2 vs 4	2-3 vs 4-3	7.45	(+/+)	1	3	3	3	3	1	1	1	1	3	2
Taiwan	3 vs 4	3-3 vs 4-3	7.71	(+/+)	1	3	3	3	3	3	3	2	3	3	2.7
Taiwan	4 vs 5	4-3 vs 5-2	174	(+/+)	3	3	2	3	4	4	2	3	3	3	3
Taiwan	4 vs 5	4-3 vs 5-3	174	(+/+)	4	4	3	3	4	3	4	3	3	3	3.4
Taiwan	6 vs 7	6-2 vs 7-3	53	(+/+)	3	3	3	3	3	3	3	3	3	3	3
Penang	1 vs 2	1-2 vs 2-3	3.29	(+/−)	4	4	4	4	4	4	4	4	4	4	4
Penang	1 vs 3	1-1 vs 3-3	17	(+/−)	4	3	4	4	4	4	4	4	4	4	3.9
Penang	2 vs 3	2-1 vs 3-1	16.4	(+/−)	4	4	4	4	4	4	4	4	4	4	4
Penang	1 vs 2	1-3 vs 2-1	3.12	(−/−)	4	4	4	4	4	4	4	4	4	4	4
Penang	2 vs 3	2-1 vs 3-2	16.2	(+/−)	3	4	4	4	4	4	4	4	4	4	3.9
Penang	1 vs 3	1-3 vs 3-3	16.7	(+/−)	4	4	4	4	4	4	4	4	4	4	4
Penang	1 vs 2	1-1 vs 2-2	30.7	(−/−)	4	4	4	4	4	4	4	4	4	4	4

Appendix 5



Appendix 5 Agarose gel electrophoresis for RT-PCR detection of TR44839 virus. RNA of five workers from each of the three additional colonies (colony 1-3, denoted as C1, 2 and 3) was extracted and used as the template for cDNA synthesis using oligo-dT primer and the strand-specific primer. cDNA was then added to the subsequent PCR reaction in which the target viral fragment was amplified. The presence of TR44839 virus is conformed if a 130 bp fragment is amplified by the PCR reaction with template cDNA synthesized using oligo-dT primer (lane 1-3), whereas active replication is evident if a 130 bp fragment is amplified by the PCR reaction with template cDNA synthesized using the strand-specific primer (lane 4-6). M, 100 bp DNA ladder; B, blank; N, negative control; P, positive control.

Appendix 6



Appendix 6 Intercolonial aggression level of colony pairs involving two colonies collected from the same site. The numbers of pairwise aggression test are 6, 21 and 9 in Okinawa, Taiwan and Penang, respectively. Different letters indicate the significant differences in aggression level between islands (p-value<0.001).