Adaptation of the brown planthopper, *Nilaparvata lugens* (Stål), to resistant rice varieties

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ABSTRACT

This thesis examines the three-way interaction between yeast-like symbionts, an insect herbivore [*Nilaparvata lugens* (Stål)] and its rice (*Oryza sativa* L.) host, during adaptation of the herbivore to resistant rice varieties. A long-term selection study (20 generations of continuous rearing, ca. 24 months) was conducted with *N. lugens* populations on four rice varieties (IR22, a susceptible variety and IR65482, IR62, and PTB33, three resistant varieties). Planthopper performance and the abundance of yeast-like symbionts (YLS) were monitored throughout the selection process. *N. lugens* populations adapted to the resistant varieties as noted by increasing body size and increased egglaying. Xylem feeding was observed as a possible behavioural adaptation of *N. lugens*: planthoppers on resistant plants had relatively high levels of xylem feeding compared with planthoppers on susceptible plants. Planthoppers selected on resistant varieties, had clear differences in YLS densities that were not related to fitness on the varieties and, therefore, did not support a YLS density-mediated adaptation hypothesis.

Furthermore, this study examined whether YLS density affected the capacity of planthoppers to switch between hosts on which they have been selected for several generations (natal plant) to new varieties (exposed plants) under normal YLS densities (symbiotic) and after reduction of YLS densities by heat treatment (aposymbiotic). The results suggested that YLS do not mediate host plant switching in planthoppers as removal of symbionts influenced body weight but not the relative capacity of nymphs to feed on different plants. This study also tested if virulence is acquired by shared feeding sites with virulent and avirulent planthoppers. In the study, planthoppers with varying levels of virulence affected the host plants differently: The most virulent hoppers appeared to suppress rice defences to a greater extent than non-virulent planthoppers. Planthoppers attained highest weights on those plants on which virulent planthoppers had previously fed which suggests that feeding by the virulent planthoppers facilitated subsequent planthopper feeding on the same plant. Our preliminary results indicate that feeding by mixed virulent-avirulent populations could potentially accelerate adaptation by *N. lugens* to resistant rice varieties.

The capacity of virulent and avirulent planthoppers to feed on a range of 24 resistant rice varieties was examined using a series of bioassays. Planthoppers were observed to feed and lay eggs on all the varieties tested, many of which have never been widely deployed in the field. Furthermore, planthoppers selected on resistant varieties often had increased fitness on other resistant varieties, even when these possess different resistance genes. However, there was no strong evidence that once planthoppers have adapted to a resistant variety, they will exhibit fitness costs on other varieties with dissimilar genes. The mechanisms underlying insect virulence are complex and further research on planthopper adaptation is necessary to help conserve genetic resources and prolong the durability of available resistant varieties.

CHAPTER 1

General Introduction: Insect adaptation to resistant varieties of agricultural crops

Jedeliza B. Ferrater

Abstract

It is estimated that worldwide yield losses due to insect damage to agricultural crops is huge. Several of these crops have resistance breeding programs in-placed for decades aimed at reducing insect damaging effects. However, the efficiency of breeding programs is limited because of the ability of the insect to overcome host plant resistance at a phenomenally rapid pace. Most studies extensively elucidated plant defenses against insect damage and in the last 50 years, research has largely focused on the search for novel sources of plant resistance. Information on the insect-side of the interaction is often largely undiscussed compared to the plant-side of the interaction. Here, we review the different strategies by which insect herbivores overcome host plant resistance with emphasis on the *Nilaparvata lugens* (Stål) and its host, rice (*Oryza sativa* L.). Several other crops which have similarly succumbed to the virulence of its insect herbivores were briefly described which suggests that insect virulence to cultivated crops is not exclusive but maybe a general occurrence. This review calls for an in-depth investigation into insect adaptation, to improve understanding of insect virulence and eventually device further measures that will significantly delay insect adaptation and increase durability of resistant crops.

Agricultural ecosystems are an ideal setting for the study of evolution in insect herbivores. The continued practice of pest management - such as the use of host plant resistance, or biological and chemical control - results in natural selection in which better adapted insects evolve to elude pest control measures (Via, 1990; Hawthorne, 1998). Several insect species have gained economic importance due to the evolution of virulence, rendering them capable of feeding on resistant plant varieties. This selection pressure has made insect herbivore adaptation to agriculturally important crops such as rice and wheat (**Table 1**) highly inevitable. Insect adaptation in this context refers to the emergence of "biotypes" or strains of insects that can damage previously resistant plant varieties (Pathak and Heinrichs, 1982; Hirae et al. 2007).

Table 1. Some examples of insect species that have adapted to host plant varieties containing novel resistance genes.

Insect Species	Insect Order: Family	Host Plant	Number of Plant Resistance Genes	Number of resistance-breaking "biotypes" identified amongst the insects
Hessian fly (*Mayetiola destructor)*	Diptera: Cecidomyiidae	Wheat	34[a]	16[b]
Russian wheat aphid (*Diuraphis noxia)*	Homoptera: Aphididae	Wheat	11[c]	5[d]
Asian rice gall midge (*Orseolia oryzae*)	Diptera: Cecidomyiidae	Rice	11[e*]	13[f]
Brown planthopper (*Nilaparvata lugens*)	Homoptera: Delphacidae	Rice	34[g]	4[h]
Green rice leafhopper *(Nephotettix cincticeps*)	Homoptera: Cidadellidae	Rice	7[g]	3[i]

Source reference – a: Garcés-Carrera et al. (2014); b: Patterson et al. (1992); c: Lapitan et al. (2007); d: Shufran and Payton (2009); e: Lu et al. (2013); f : Lima et al. (2007); g: Fujita et al. (2013); h: Rahman et al. (2009); i: Fujita et al. (2010); *: major genes characterized; more uncharacterized genes.

There is no single definition of a "biotype" (Shufran and Payton, 2009) but several authors agree on the following points: 1) "biotypes" are intraspecific categories; 2) "biotypes" are usually morphologically indistinguishable and 3) "biotypes" differ in expressed biological attributes (Eastop, 1973; Gallun and Khush, 1980; Diehl and Bush, 1984; Saxena and Barrion, 1987). The occurrence of resistance-breaking insect "biotypes" has already been observed more than 100 years ago (*Halesidota* spp.: first mentioned by Walsh, 1864, see Shufran and Payton, 2009). The potential development of herbivore populations that kill or damage resistant crops complicates pest management programs (Hawthorne, 1998; Shufran and Payton, 2009). Therefore, evolution, in terms of adaptation to resistant crop varieties, represents a major cost for agriculture. Simms (1987) has encouraged ecologists to study the ability of insects to overcome crop protection measures from an evolutionary perspective. Ecologists and evolutionary biologists must learn how to reduce the potential for insect pests to adapt to resistant varieties.

Adapted populations and "biotypes"

The use of the term "biotype" has elicited intensive debate (Shufran et al. 2007). Claridge and den Hollander (1983) argued that the continued use of this term only leads to confusion and further compromises our understanding of the breakdown of host plant resistance. The specific labeling of biotypes by numbering or naming is not appropriate for the following reasons: (1) the biotype populations are highly variable and even show individual variation for virulence; and (2) there is little genetic homogeneity in virulence characteristics within biotypes which are likely controlled by many genes. These suggestions arose from selection studies conducted by Claridge and den Hollander (1982) where the authors found that the brown planthopper *Nilaparvata lugens* Stål (Homoptera: Delphacidae) "biotypes" 1, 2 and 3 reared at the International Rice Research Institute (IRRI) could be

converted from one biotype to another within about ten generations on a particular cultivar. Up to the present, however, the term "biotype" is still actively used and no new term has been unanimously accepted to replace it. In this thesis, I use the term in quotation marks (i.e., "biotype") to refer to an insect population that has adapted to previously resistant plants.

Another potential cause of confusion in discussions about co-adaptation between host plants and pest insects is that the loss of effectiveness of plant defense is often erroneously described as 'breakdown' of host plant resistance. However, as indicated by Claridge and den Hollander (1980), the resistance has not been broken down; it has simply become ineffective against the new forms of the insect. For example, evaluation of the adaptation of a gall midge to the resistant rice variety Suraksha, revealed through proteomic analyses that the gall midge resistance gene *Gm1* still functioned, but that resistant gall midge populations were no longer affected by the gene products (Sinha et al. 2012). In another study, the *Nr*-gene in lettuce, *Lactuca sativa* L. (Asteraeae) became ineffective after 20 years of conferring resistance against the specialist black currant lettuce aphid, *Nasonovia ribis-nigri* (Mosely)(Hemiptera: Aphididae) (Ten Broeke et al. 2014).

Due to the eventual adaptation of herbivorous insects on resistant host plants, intense screening of resistant donor varieties and the incorporation of resistance traits into susceptible, high yielding varieties through marker-assisted selection has been the primary focus of modern plant breeding. In rice, screening of varieties for resistance against major 'hopper' pests (Delphacidae and Cicadellidae) began in the 1970s. To date, over 80 resistance genes and Quantitative Trait Loci (QTL) have been identified (Fujita et al. 2013). Several of these genes have been incorporated into Near-Isogenic Lines (NILs) to help develop resistant rice varieties (Sharma et al. 2004). However, screening studies conducted throughout South and South-East Asia indicate that only a few of these genes are currently effective in reducing

N. lugens fitness (Myint et al. 2009a,b; Horgan, 2012; Fujita et al. 2013). It has been proposed that varieties containing several minor genes are more durable than monogenic varieties with major genes. Traditional varieties are said to contain a suite of minor resistance genes which contribute to their inherent resistance (Alam and Cohen, 1998).

Gene pyramiding for durable resistance

Recently, "gene pyramiding" has been proposed as a potential strategy to increase the durability of resistance in rice varieties. Pyramiding is expected to delay the onset of insect adaptation by incorporating two or more resistance genes into a single variety (Sharma et al. 2004; Liu et al. 2009). However, the development of resistant varieties using gene pyramiding has produced variable results. Myint et al. (2012) evaluated the resistance of NILs, including a pyramid line, with *BPH25* and *BPH26* genes against *N. lugens*. Their study indicated that the pyramided line (*BPH25+BPH26*) was resistant against planthoppers from East Asia whereas the monogenic lines (*BPH25* and *BPH26*) were not. In another study, pyramiding the *bph20*(t) and *Bph21*(t) resistance genes in rice failed to increase resistance against South Vietnamese strains of *N. lugens* (Fujita et al. 2009). Similarly, Vu et al. (2014) reported that the additive effects of two resistance genes (*Grh2+Grh4*) in pyramided lines significantly increased mortality of nymphs and adults of the green rice leafhopper, *Nephotettix cincticeps* Uhler (Hemiptera: Deltocephalidae) compared with monogenic NILs. In this case, the pyramided NIL also reduced the capacity of the adult leafhoppers to lay eggs (Vu et al. 2014). In contrast, in a greenhouse bioassay, Sharma et al. (2004) found that a pyramided rice line containing the *Bph1* and *Bph2* resistance genes against the brown planthopper performed similarly to a monogenic resistant line (*Bph1*).

Therefore, although pyramided lines can enhance resistance to planthoppers and leafhoppers, caution should be taken in the deployment of these pyramided varieties since it

is unknown whether pyramided lines could lead to a more rapid adaptation against the resistance genes they contain than if the genes were sequentially deployed as monogenic varieties (Fujita et al. 2013). However, this will all be dependent on the mechanisms of resistance of the different genes – if similar, breakdown is easier than when very different mechanisms are involved.

How do herbivores overcome plant defenses?

Over the past 30 years, great advances in the understanding of plant defenses against insect herbivores have been made (Howe and Jander, 2008; War et al. 2012). The protective mechanisms that plants use range from simple passive defenses in the form of physical barriers such as trichomes and waxy layers, to more sophisticated active chemical defenses that are synthesized upon recognition of insect or pathogen intrusion (Kessler and Baldwin, 2002; Kaloshian, 2004; Schoonhoven et al. 2005; Handley et al. 2005; Howe and Jander, 2008; Mithöfer and Boland, 2012). In addition, host plant chemistry, specifically the composition of plant secondary metabolites, is known to limit the fecundity and reproductive performance of insect herbivores (Awmack and Leather, 2002; Schoonhoven et al. 2005). Advances in molecular biology, biochemistry and ecogenomics have elucidated the plant defense signaling pathways and the direct and indirect plant defense mechanisms that are activated upon insect wounding (Kessler and Baldwin, 2002; Dicke et al. 2003; Mewis et al. 2005; Kaloshian and Walling, 2005; Thompson and Goggin, 2006; Bruce and Pickett, 2007; Kant and Baldwin, 2007; Zheng and Dicke, 2008; Dicke et al. 2009). It is now known that plants produce toxins and defensive proteins which affect the insect's physiology. In addition, upon herbivory, plants emit volatiles that attract insect predators (Howe and Jander, 2008; Dicke et al. 2009; Dicke and Baldwin, 2010) which significantly influences top-down and

bottom-up tritrophic interactions among plants, herbivores and their natural enemies (Ode, 2006; Bruce and Pickett, 2007; Inbar and Gerling, 2008; Dicke and Baldwin, 2010).

A recent quantitative mass spectrometry-based proteomic approach has been used to compare the protein expression profiles of leaf sheaths of a susceptible rice variety (TN1) and a resistant line carrying a *BPH15* gene, both in response to infestation by the brown planthopper. Both sets of rice line shared the same response which appeared stronger in the susceptible line compared with the resistant line. The expression of Jasmonic Acid (JA) synthesis proteins, oxidative stress proteins, beta glucanase Gns1, protein kinases and clathrin (protein that plays an important role in the onset of basal defense responses) increased in both lines, but higher expression levels were seen in the susceptible line after attack by *N. lugens*. In contrast, beta glucanase Gns5 expression remained unchanged and the glycine cleavage system H-protein was up-regulated only in the resistant line. Beta glucanases play a role in plant defense and development. These differences may possibly be attributed to a difference in the levels of damage inflicted on the susceptible and resistant lines, as well as differences in the plant genotypes (Wei et al. 2009).

Herbivores have to deal with a huge arsenal of plant defenses in order to survive and reproduce (Jansen et al. 2009; Kessler and Baldwin, 2002; Schoonhoven et al. 2005). Generally, herbivores employ offensive strategies to match plant-imposed challenges. Herbivore offenses are traits which allow them to increase feeding and other exploitation of the host plant. These strategies range from the least aggressive behavioral (feeding and oviposition) choices to more aggressive morphological and physiological offenses as well as in the interference with signal transduction pathways (Karban and Agrawal, 2002; Kempema et al. 2007; Kant et al. 2008). The whole repertoire of insect offenses to successfully exploit the host-plant defensive strategies is, however, not as actively explored as the plant-side of

the story (Karban and Agrawal, 2002). However, there have been a number of recent studies that provide interesting insights into interference with plant defensive traits by phloem feeding insects (Will et al. 2007; Kempema et al. 2007; Zhang et al. 2009). Nevertheless, to date, knowledge on herbivore manipulation or tactics to evade host plant defenses remains limited (Musser et al. 2002; Felton and Tumlinson, 2008; Walling, 2008).

Behavioral (feeding and oviposition) choices: The first and least aggressive offensive trait employed by herbivores to exploit their host plant is choice (feeding and oviposition) (Karban and Agrawal, 2002). An insect's preference for a specific food type is governed by innate and learned behaviors (Papaj and Prokopy, 1989; Jaenike, 1990). Natural selection has favored herbivores that are selective about the food they ingest (Karban and Agrawal, 2002). Recent findings indicate that many insects rely heavily on learning in behaviors including feeding and oviposition, which are associated with their fitness (Dukas, 2008; Behmer, 2009).

Since a herbivorous insect must find, select, and then successfully use a host plant, the process of insect adaptation to a new host plant will eventually involve changes in behavioural (feeding and oviposition preference) attributes of the insect (Hawthorne, 1999). The brown planthopper is attracted to the color green, high humidity and the odor of rice plant extracts (Saxena and Pathak, 1979; Foster et al. 1983). In contrast, whiteflies use predominantly color, while aphids use both visual and olfactory cues to determine the value of the plant as a feeding and oviposition host (Walling, 2008). Upon landing on the plant, insect adults evaluate the tactile and chemical characteristics of the plant surface to determine its suitability as food, shelter and/or for oviposition (Walling, 2008; Schoonhoven et al. 2005). In planthoppers, a wide variety of peripheral sensory structures are associated with the stylets, labium, precibarium, and tarsi which provide information concerning the acceptability

of the host plant (Cook and Denno, 1994). These morphological features, combined with the secretion of small amounts of saliva to dissolve surface chemicals and imbibe liquids at the surface allow insects to sample the chemical defenses in the phyllosphere (Miles, 1999) and to critically appraise the host or non-host status of the plant (Muller and Riederer, 2005). For example, after surface exploration by the brown planthopper, chemical cues from the surface waxes of the resistant rice variety IR46 reduce the insect's settling and probing compared with the susceptible variety, IR22 (Woodhead and Padgham, 1988).

Planthoppers require sucrose for their metabolism. Glucose, fructose and maltose also serve as nutrients in addition to sucrose (Koyama, 1985). Comparative studies have shown that susceptible and resistant rice varieties contain the same amounts of sugar but that planthoppers ingest more sugar from susceptible cultivars: this suggests the action of antifeedants in the resistant varieties which inhibit insect feeding activity (Jung and Im, 2005). Also, asparagine and sulfur-containing amino acids appear to have stimulatory effects on planthopper feeding: planthoppers prefer varieties (and individual plants) with higher asparagine content (Sogawa and Pathak, 1970; Shigematsu et al. 1982).

Generally, insect offspring will thrive on a good host whereas an insect population will decline on a poor host (Walling, 2008). There is some evidence that mobile ovipositing females assess different host plant species and place their eggs on those hosts with potential to result in the highest performance such as in the case of *Cephaloleia* where beetle oviposition preferences follow predictions based on the "mothers knows best" principle. The "mother knows best" principle suggests that females prefer to lay eggs on hosts that increase offspring survival (Scheirs et al. 2000; Garcia-Robledo and Horvitz, 2012), but this is not entirely true for all species (Karban and Agrawal, 2002) because some females will oviposit

on plants that increase adult longevity but reduce offspring survival, as predicted by the "optimal bad motherhood" principle (Scheirs et al. 2000; Garcia-Robledo and Horvitz, 2012).

Morphological specialization: Morphological traits affect the ability of insects to exploit their host plants. Comparative studies have revealed a significant association between the morphology of the feeding apparatus and ingested food type, indicating the specific offensive strategies of herbivores (Karban and Agrawal, 2002). For example, some species have typical incisors for tearing and chewing relatively soft foliage while other species have toothless snipping mandibles for cutting larger, tougher grasses or the mature leaves of trees (Bernays and Janzen, 1988). The plant sap-sucking insects of the order Homoptera display a sophisticated level of specialization on plant parts which is possible because of their long and narrow proboscis that is utilized to navigate plant tissues and evade plant allelochemicals (**Figure 1**) (Tjallingii and Hogen Esch, 1993; Bernays and Chapman, 1994). Many species of aphids and planthoppers feed on plant phloem, while other species concentrate their feeding on the xylem bundles or other specific plant tissues (Bernays and Chapman, 1994; Pompon et al. 2010).

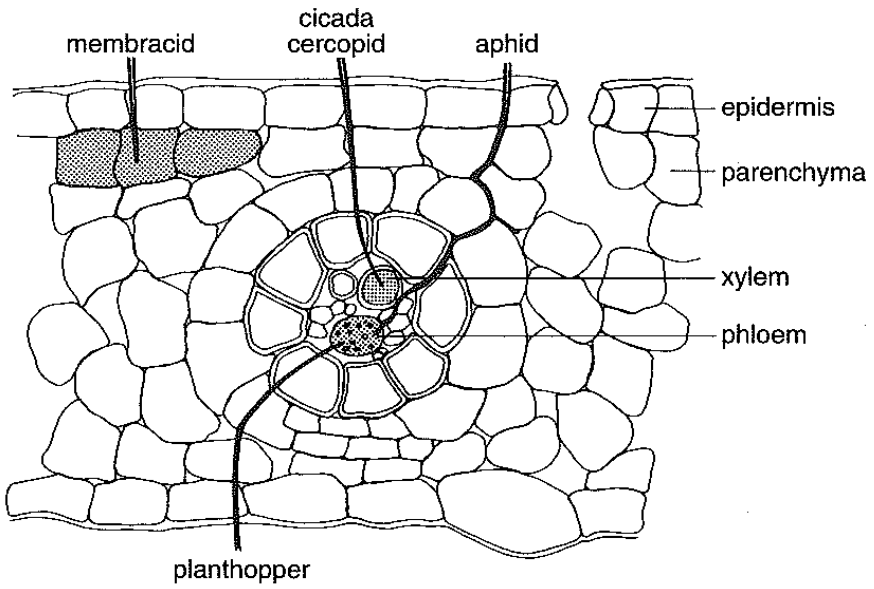

Figure 1. Cross section of a leaf showing specific tissues which plant-sucking insects generally feed upon (Bernays and Chapman, 1994).

Aphids and whiteflies are thought to disguise themselves and deceive their hosts using their stylets to deliver salivary chemicals and/or proteins into the plant via the salivary sheath (Walling, 2008). The brown planthopper secretes saliva that is similar to aphid saliva but possesses an additional sheath with protuberances on the outer surface. This is thought to be an adaptive mechanism for the insect to reduce friction within rice tissues and make the ingestion pathway smooth (Wang et al. 2008).

When the insect's stylet pierces the phloem, the plasma membrane must be sealed to avoid leakage of the phloem sap to the apoplast. Plants repair insect–induced lesions by depositing callose and complex forisomes (complex proteins that need calcium ions to be structurally functional) to heal the wounds (Will and van Bel, 2006; Furch et al. 2008). This wound healing event can accidentally block the insect's food canal. Hemipterans (e.g., aphids) antagonize this event by sealing the lesion with sheath saliva which contains calcium

ion-binding proteins that prevent the formation of forisomes. It is presumed that saliva of other phloem-sap feeders performs the same role (Miles, 1999; Will et al. 2007).

In a recent study, using the electrical penetration graph recording technique, the brown planthopper which ingests phloem sap through its stylet mouthparts, spent more time wandering but less time ingesting sap on plants with resistance genes than on susceptible plants. *N. lugens* feeding up-regulated callose synthase genes and induced callose deposition in sieve tubes at the stylet insertion site that prevented *N. lugens* from ingesting the phloem. The callose remained compact in resistant rice plants. However, on susceptible plants, *N. lugens* feeding also activated another gene which codes for ß-1,3-glucanase that degrades the deposited callose leading to resumed feeding of the *N. lugens* from the phloem (Hao et al. 2008).

Insects exhibit phenotypic plasticity, changing their phenotype over time to adapt to changes in the environment (Karban and Agrawal, 2002). Some organisms undergo morphological alterations in response to diet or to a new habitat which may be temporary or fixed (Fordyce, 2006). Plasticity contributes considerably to life history diversity within the species and although reports are rare, plasticity is thought to influence the development of "biotypes" (Leclaire and Brandl, 1994).

Enzymatic detoxification of plant toxins: Herbivores must deal with a large array of toxic plant chemicals that are detrimental to their cellular processes (Duffrey and Stout, 1996). The capacity of the insect to metabolize and degrade toxic chemicals is important for its survival. Herbivorous insects express their detoxification capacity in response to plant chemical selection pressures (Terriere, 1984; Brattsen, 1988). To date, 27 insect species from various insect groups such as Lepidoptera, Diptera, Orthoptera, Heteroptera, Coleoptera (Yu and Hsu,

1993) and Homoptera (Ramsey et al. 2010) as well as mites have been shown to possess inducible detoxification systems.

Most studies on the mechanisms of tolerance of insects to allelochemicals have focused on enzymes which directly detoxify the allelochemicals (Yu et al. 1979; Yu, 1983; Ahmad et al. 1986; Larson, 1986; Ahmad, 1992; Behmer, 2009). Insects respond to plant secondary metabolites through a range of mechanisms including avoidance of those plant tissues that contain the constitutive defences of the plants, through enzyme target site insensitivity, the rapid passage of toxins through the gut; efflux pump mechanisms that move toxic substances out of the cell, and through direct metabolic detoxification (Li et al. 2007; Ramsey et al. 2010). Some insects can act on allelochemicals by sequestration, temporarily storing them in specific tissues to be used to defend themselves against predators (Gols and Harvey, 2009; Muller, 2009).

There are three major classes of enzymes that have been associated with the detoxification process. These can also confer resistance to insects against xenobiotics (allelochemicals and insecticides) (Li et al. 2007). Detoxification is mediated through esterases, glutathione-S-transferases (GSTs) and cytochrome P-450 monooxygenases (P450s) (Ramsey et al. 2010). The insect species that were assayed for these enzymes and the mechanisms of action of each are presented in **Table 2**.

The system of detoxification of plant defence chemicals (allelochemicals) by insects is different from that of detoxification of insecticides (Berenbaum, 1995). Insecticides tend to be applied as pure compounds, whereas allelochemicals occur in mixtures of structurally different classes of compounds. Allelochemicals can influence food selection behaviour (non-preference), can bring about growth reduction, or act as toxins that kill the insect (Berenbaum, 1995).

Table 2. List of insect enzymes that are involved in the detoxification of plant allelochemicals (from Li et al. 2007; Ramsey et al. 2010).

Enzyme groups	Insect species	Mechanism of Action
Cytochrome P-450 monooxygenases (P450s)	Black swallowtail *(Papilio spp.*), fruit fly (*Drosophila spp.*), tobacco hornworm (*Manduca sexta*), parsnip webworm (*Depressaria pastinacella*), corn earworm (*Helicoverpa zea*), green peach aphid (*Myzus persicae*)	Oxidative detoxification
Glutathione S-transferases (GSTs)	fall armyworms (*Spodoptera frugiperda, Trichoplusia ni, Anticarsia gemmatalis*), spruce budworm *(Choristoneura fumiferana*), tobacco hornworm (*Manduca sexta*), Hessian fly (*Mayetiola destructor*), pea aphid (*Acyrthosiphon pisum)*	Conjugation of xenobiotics to the thiol group of reduced glutathione
Esterases	tiger swallowtails (*Papilio glaucus, P. canadensis*), tobacco cutworm (*Spodoptera litura*), gypsy moth (*Lymantria dispar*), pea aphid (*Acyrthosiphon pisum*)	Biochemical hydrolysis

Ramsey et al. (2010) studied the level of detoxification enzymes in the specialist pea aphid, *Acyrthosiphon pisum* (Harris), which feeds almost exclusively on plant species in the family Fabaceae, to that in the generalist green peach aphid, *Myzus persicae* (Sulzer), which feeds on hundreds of species from over 40 plant families. The results were consistent with the hypothesis that a generalist phloem-feeding insect herbivore requires a greater number of detoxification enzymes than a specialist herbivore. Comparison of the *M. persicae* cDNA and *A. pisum* genomic sequences revealed that at least 40% more cytochrome P450 genes are found in *M. persicae* than in *A. pisum* (Ramsey et al. 2010). In contrast, there were no major differences between the two species in the numbers of glutathione *S*-transferases, and carboxy/cholinesterase genes. Furthermore, Ramsey et al. (2010) indicate that because the

available cDNA data for *M. persicae* is incomplete, these identified detoxification genes appear to be an underestimation of the actual number of genes responsible for detoxification of allelochemicals in *M. persicae*.

Allelochemical Sequestration: To successfully colonize plants that possess toxic compounds, some herbivores sequester secondary phytochemicals into specialized tissues or glands (Duffey, 1980). Sequestration is considered to be an offensive trait that requires several conditions to be met: (1) the insect must be able to consume the host allelochemicals (2) the herbivore must be relatively tolerant of the plant secondary chemicals, (3) the herbivore must ingest the chemicals without metabolizing them, and (4) the herbivore must store the chemicals in specific tissues (Dobler, 2001).

An example of sequestration is in the flea beetle genus *Longitarsus* (Coleoptera: Chrysomelidae) which sequesters plant pyrrolizidine alkaloids and iridoid glycosides into their tissues (Dobler, 2001). Several butterflies and moths sequester toxic substances such as terpenes and phenols from their host plant rather than investing in the synthesis of defense chemicals, and use these compounds against predators (Nishida, 2002). The larvae of the pipevine swallowtail *Battus philenor*, sequester toxic alkaloids which increase larval survival (Fordyce and Nice, 2008). The cabbage aphid *Brevicoryne brassicae* sequesters glucosinolates (sinigrin), which are particularly important for wingless aphids that are committed to a sedentary life and have a limited ability to escape predation (Kazana et al. 2007).

Insect Symbionts: It is increasingly clear that patterns of plant and herbivore association cannot be understood by just considering plant-herbivore interactions alone. Several insect species from different taxonomic groups harbor maternally transmitted endosymbionts (**Table 3**) (Douglas, 2009; Tang et al. 2010; Frago et al. 2012; Ferrater et al. 2013).

Herbivores harbor microbial symbionts e.g., bacteria, yeasts, rickettsia (Noda and Kawahara, 1995; Frago et al. 2012; Ferrater et al. 2013; Douglas, 2014) that allow them to exploit their host plants more effectively (Karban and Agrawal, 2002; Frago et al. 2012; Ferrater et al. 2013; Douglas, 2014). Symbiosis is receiving increased attention among all aspects of biology because of the unifying themes it helps to construct across ecological, evolutionary, developmental, semiochemical, and pest management theory (Klepzig et al. 2009).

One of the best studied examples of symbiosis concerns the aphid *Myzus persicae* which is a generalist phloem-feeder that has a symbiotic relationship with the γ-proteobacterium of the genus *Buchnera.* This bacterium is an obligate symbiont of aphids. The aphid benefits from the association because *Buchnera* provides essential amino acids and also produces type III protein effectors that are present in the salivary secretions and function to suppress plant defense responses upon puncturing plant cells with the insect stylet (Alvarez, 2007). The phytophagous leaf-mining moth *Phyllonorycter blancardella* has an interesting association with its bacterial endosymbiont (possibly *Wolbachia*). Here, the bacterial symbiont is implicated in insect manipulation of the host plant. This association allows the insect to manipulate the physiology of its host plant, resulting in the 'green-island' phenotype (photosynthetically active green patches in otherwise senescing leaves) which ensures successful development and an adequate food supply during senescent stages of the plant (Kaiser et al. 2010).

Table 3. Survey of microbial symbionts of insects (haematophages not included).

Insect	Microorganisms
(A) General feeders	
Blattidae (cockroaches)	*Blattabacterium* (flavobacteria)
Mallophaga (biting lice)	Not known
Psocoptera (book lice)	*Rickettsia* sp. (α-proteobacteria)
Coleoptera*, e.g. Weevils Various	γ-proteobacteria
Anobiid beetles	*Symbiotaphrina* (fungi)
Hymenoptera	
Camponoti (carpenter Ants)	*Blochmannia* (γ-proteobacteria)
(B) Plant sap feeders	
Hemiptera	
Auchenorrhyncha (e.g. leafhoppers, plant-hoppers)	*Baumannia cicadellinicola* (γ-proteobacteria); *Sulcia muelleri*; *Rickettsia; Firmicutes, Actinobacteria, Bacteroidetes*; Yeast-like (Pyrenomycete-Ascomycotina) (Bacteroidetes); Clavicipitacean fungi in some plant-hoppers
Aphids	*Buchnera aphidicola* (γ-proteobacteria) or clavicipitacean fungi
Whitefly	*Portiera aleyrodidarum* (γ-proteobacteria)
Psyllids	*Carsonella ruddii* (γ-proteobacteria)
Scale insects	*Tremblaya principes* (β-proteobacteria)

Research has shown that microorganisms can play a role as unseen mediators in the utilization of plants by insect herbivores and in making plants suitable hosts to herbivores (Krischik and Jones, 1991; Dicke, 1996; Schoonhoven et al. 2005; Frago et al. 2012; Ferrater et al. 2013; Douglas, 2013, 2014). Specifically, symbionts enable herbivores to utilize foods that the herbivores cannot normally digest, supplement the less nutritious and unbalanced diets of phloem-feeding herbivores, and aid herbivores in utilizing otherwise toxic foods and in weakening or overcoming the host plant's defenses by detoxification (Karban and Agrawal, 2002; Douglas, 2006; Douglas, 2013). The acquisition of these symbionts by the

insect can be considered a key innovation in the evolution of the herbivore (Moran and Telang 1998; Ferrater et al. 2013).

The rice brown planthopper is known to harbor Yeast-Like Symbionts (YLS) (Noda, 1974; Noda, 1977; Chen et al. 1981a,b; Shankar and Baskaran, 1985; Koyama, 1985; Shankar and Baskaran, 1988; Ferrater et al. 2013). YLS have been recorded in the fat body of the abdomen of nymph and adult *N. lugens* as well as in the eggs (Noda, 1974; Nasu, 1981). Scanning and transmission electron microscopy has confirmed the ultrastructure of the symbionts as bacilliform shaped, containing 2 layers of cell walls and with reproduction by budding (Cheng and Hou, 1996). The symbionts are transmitted to the next generation of planthopper through the ovary as a mass of organisms which is called a symbiont ball that is initially located at the posterior pole of the *N. lugens* egg. The symbiont ball moves to the anterior end as the embryo develops (Nagamine et al. 1993).

The mechanism of YLS transmission from the fat body to the oocyte has been revealed by both light and electron micrographs. The symbionts in the mycetocytes move out of the syncytium (formed from a layer of fat body cells) by exocytosis, and are released into the hemocoel. The free YLS in the hemolymph then move to the ovarioles near the pedicel which is enclosed by follicle cells. Then, the symbionts enter the follicle cells around the primary oocyte by endocytosis at the epithelial plug of the ovariole. The YLS then assemble at the posterior end of the mature egg forming the symbiont ball (Cheng and Hou, 2001).

It has been suggested that YLS supply their host with proteins for normal embryonic and postembryonic development (Noda et al. 1979; Lee and Hou, 1987). Uricase enzyme produced by YLS plays a key role in recycling *N. lugens* uric acid waste products into essential amino acids. These amino acids complement the limited nutrients present in the rice phloem sap (Sasaki et al. 1996; Hongoh and Ishikawa, 1997). Phylogenetic analysis on the

uricase genes present in YLS of the brown planthopper has placed the YLS in the Ascomycota group. Direct sequencing of the 18s rDNA of YLS of *N. lugens* has further placed YLS in the class Pyrenomycetes in the subphylum Ascomycotina (Noda et al. 1995).

Electrophoretic karyotyping of YLS in *N. lugens* has revealed the probable chromosome number as 4, and the tentative genome size as 17.3 Megabase pairs (Mbp). The organization of the chromosomes as well as the genome size is similar to that of non-symbiotic yeasts and fungi (Noda and Kawahara, 1995).

Role of symbionts in adaptation

Lu et al. (2004) have correlated YLS abundance to the virulence of *N. lugens* on resistant rice varieties. Their results indicated that YLS counts were positively correlated with the virulence of *N. lugens*. They strongly implied that YLS played a crucial role in virulence shifts among populations of *N. lugens*: YLS counts on *N. lugens* were lowest in the second generation fed on resistant varieties but showed a marked increase in abundance by the fourth generation as *N. lugens* began adapting to the resistant rice varieties (Lu et al. 2004).

In another study, Chen et al. (2011) found changes in the amino acid composition of *N. lugens* after several generations of selective rearing. Total nitrogen content and the concentration of rare amino acids increased, but some common amino acids decreased over successive generations (Chen et al. 2011). Examination of the planthopper colonies during two generations of selection (the eight and eleventh) revealed that YLS improved nymphal performance in the eighth generation, but appeared to be a drain on nymphs by the eleventh generation [as shown by the higher performance of aposymbiotic (where symbiont densities were reduced) nymphs than normal, symbiotic nymphs in the eleventh generation](Chen et al. 2011).

Tang et al. (2010) found that the composition and abundance of bacterial symbiont OTUs differed among populations of *N. lugens* reared on three rice varieties (TN1, Mudgo and ASD7). Tang et al. (2010) suggested that theses bacterial symbionts may also mediate virulence adaptation and this may be associated with changes in bacterial community composition and function.

Insect symbionts have also been implicated in the detoxification of plant toxins as well as synthetic compounds such as insecticides (Dicke, 1988; Barbosa et al. 1991). A study on the cigarette beetle, *Lasioderma serricone* (Coleoptera: Anobiidae) has demonstrated a localized detoxification activity of the allelochemicals, 1-naphthyl acetate and tannins in the mycetosomes which assist the insect in surviving on toxic dried plant substrates including tobacco, straw, seeds, and pepper (Dowd, 1989). In another study, *Lasioderma serricone* beetles rendered free of yeast symbionts (aposymbiotic) had high mortality when treated with representative plant defense toxins compared with control insects whose symbionts had remained untreated (Dowd and Shen, 1990).

History and status of the brown planthopper problem

The brown planthopper has been a serious pest of rice in Korea and Japan for centuries, but only emerged as an important pest of tropical rice about 60 years ago. The planthopper occurs in most tropical countries in Asia and the South Pacific including the Philippines, India, Malaysia, Vietnam, Thailand, Fiji, the Solomon Islands, Bangladesh, Sri Lanka, Taiwan and Indonesia (IRRI, 1979). Brown planthopper damage has been observed sporadically in rice-growing regions of Asia since the late 1950s to 1960s, but a peak of damage due to high-density infestations of this insect pest on rice fields was observed in the 1970s where the total estimated yield loss across many countries was almost US$300 million (IRRI, 1979).

A condition called hopperburn, characterized by severe browning and drying up of plants in localized patches or across entire rice fields occurs after sustained sucking and feeding by the brown planthopper. Moreover, apart from the large-scale physiological or mechanical damage, the insect also causes indirect damage because it is a vector of both ragged stunt and grassy stunt viruses which reduce yield (Hibino, 1996).

In the last 50 years, host plant resistance has been the primary research focus of the IRRI for *N. lugens* management (Fujita et al. 2013). Over 34 planthopper resistance genes and QTLs have been identified by breeders for use in the development of resistant rice varieties. Some of these genes have been pyramided in NILs through marker-assisted selection to develop more durable resistance against the planthopper (He, 2007; Fujita et al. 2013). This approach, however, may not be sufficient because of the ability of planthoppers to adapt rapidly to resistant varieties and become virulent to novel plant genotypes (Roderick, 1994).

In 2005, the brown planthopper, which had remained at low densities since the 1980s, occurred in a series of outbreaks in Vietnam, China, Korea and Japan. Large-scale outbreaks have continued to occur in South- and South-East Asia with the largest losses recorded in China in 2008, where 7.5 million hectares of rice in several provinces were infested resulting in a loss of 2.8 million tons of rice. In Vietnam, in addition to *N. lugens* damage, crops were severely infected with viral diseases transmitted by the insect (Heong, 2008, 2009). In 2006, the Vietnamese Ministry of Agriculture reported a loss of 400,000 tons of rice, which prompted the government to restrict exports for fear of domestic shortages (Heong and Escalada, 2008). Since 2009, *N. lugens* has continued to be a widespread problem for rice growers with outbreaks documented in China, Thailand, the Philippines, India, Malaysia, Vietnam, Indonesia and Bangladesh (Bottrell and Schoenly, 2012).

Life history strategy of the brown planthopper

The brown planthopper belongs to the large group of phytophagous planthoppers in the order Homoptera. Of the 19 identified families of planthoppers within the superfamily Fulgoroidea, the Delphacidae has received most research attention because it represents a number of important sap-feeding pests of agricultural crops (Denno and Perfect, 1994).

The adult insect is brownish in color and about 2.0-3.5 mm in length. On average, oviposition by *N. lugens* of tropical origin starts 3 days after adult emergence while it takes longer among subtropical and temperate populations (Wada et al. 2007). **Figure 2** shows the life cycle of *N. lugens*. At warm (30^{o}C) temperatures, eggs and nymphs develop more rapidly but rates of survival are reduced compared to cool (10^{o}C) temperatures. For *N. lugens*, individuals develop fastest between 25 and 28^{o}C. Variations in temperature in either direction result in delayed development (Denno and Roderick, 1990). At 27^{o}C, it takes about 4-8 days for eggs to hatch into nymphs.

Nymphs develop through five instars within a period of 14 days, before emergence to adulthood. First instar nymphs move to the leaves when feeding while late instars prefer the basal part of the plant. Adult longevity is 11-21 days and the adult stage infests rice during the maximum tillering stage (Manjumath, 1977; Reissig et al. 1986). *N. lugens* are r-strategists being both highly fecund and mobile (Denno and Roderick, 1990).

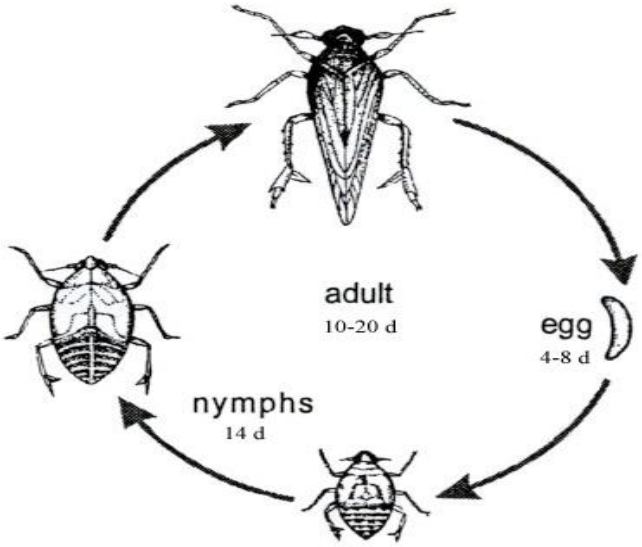

Figure 2. Life cycle of the rice brown planthopper, *Nilaparvata lugens* at 27^{o}C (Reissig et al. 1986).

N. lugens exhibits wing dimorphism, having macropterous and brachypterous forms (Sogawa, 1982). The macropterous form has well-developed wings which allow the hoppers to travel distances of up to 1000 km by transoceanic migration (Denno and Roderick, 1990). Upon host-plant quality deterioration and increased planthopper population density, which can greatly reduce fitness, brown planthoppers move to search for other habitats (Denno and Roderick, 1990). In contrast, the brachypterous form is more sedentary, tends to reproduce earlier, and is more fecund than its macropterous conspecific, which suggests that there is a trade-off between flight and reproduction (Kisimoto and Rosenberg, 1994). Such a trade-off has been reported for many insect species (Zera and Denno, 1997).

Planthopper feeding

The brown planthopper is monophagous on rice (Mochida and Okada, 1971; Sogawa, 1982). An extensive review of the mechanisms of feeding in the brown planthopper was published by Sogawa (1982). The feeding process involves four major steps: 1) orientation to the host plant; 2) labial exploration; 3) stylet probing; and 4) sucking.

Host orientation is stimulated by a number of volatile compounds in the rice plant that are perceived through the antennae. This is followed by sampling by *N. lugens* on the plant surface with its labium to determine which areas are easy to penetrate with its stylet. Prior to stylet probing, *N. lugens* secretes coagulative saliva that will make a tight connection between the stylet and the plant epidermis. Probing continues and the stylet seeks the phloem, which is the target site for feeding. Recently, the ultrastructure of the salivary sheaths produced by the *N. lugens* on rice tissues and artificial media revealed that *N. lugens* prefers the thick part of the first two outer leaf sheaths for probing and ingestion (Wang et al. 2008). A sustained feeding event is concentrated in the phloem and in the process degrades the cytoplasm and its components due to ingestion of the phloem cytoplasmic components (Sogawa, 1982; Wang et al. 2008).

Successful feeding on the phloem entails overcoming the phloem responses upon insect wounding. Phloem releases coagulating proteins that clog the sieve tubes in the phloem and the capillary food canal in the stylet (Tjallingii, 2006). Stylet penetration of the phloem by insect herbivores can be monitored using the electric penetration graph (EPG) technique (Tjallingii, 1988). EPG signals have been correlated with aphid feeding activity and the tissue location of the stylet tips. In several studies, EPG parameters have been used to obtain information to locate resistance factors in host plant tissues (Alvarez, 2007). Velusamy and Heinrichs (1986) first investigated the feeding activity of *N. lugens* using an electronic recording device on three resistant cultivars and one susceptible variety. Waveforms for probing, salivation and ingestion showed that *N. lugens* probed repeatedly, salivated longer and ingested for a shorter period on a resistant variety than on a susceptible variety.

Seo et al. (2009) evaluated the stylet penetration behavior of *N. lugens* on the susceptible variety TN1 and classified EPG signals into seven waveforms: 1) no penetration;

2) penetration initiation; 3) salivation and stylet movement; 4) extracellular activity near the phloem region; 5) intracellular activity in the phloem region; 6) phloem sap ingestion; and 7) activity in the xylem region.

To understand the development of the resistance-breaking ability of *N. lugens* populations in Korea, Seo et al. (2010) conducted nymphal survivorship tests and EPG analysis on four populations of *N. lugens*, one collected in the 1980s, and three from the 2000s (2005, 2006 and 2007) using resistant rice varieties containing either *Bph1*, *bph2* or *Bph3* resistance genes. EPG analysis revealed that the ratio of *N. lugens* that could reach the phloem sap ingestion waveform after 15h on resistant varieties was higher in the current *N. lugens* populations (2005-2007). The current populations also showed high resistance-breaking ability on the resistant varieties by their elevated survival rates. However, the current populations showed significantly longer pre-reaching time from the start of penetration to the first ingestion waveform. These results suggest that although the current Korean *N. lugens* populations showed a high resistance-breaking ability through increased survival rates on resistant varieties, they still encountered difficulties in feeding on the phloem sap of resistant varieties (Seo et al. 2010).

General research hypotheses

Previous studies on the mechanisms of insect-plant interactions have focused mostly on plant responses to insect attack. Recently, there have been several molecular-based studies that addressed the processes taking place upon insect herbivory. In contrast, herbivore adaptation and feeding mechanisms on resistant plant varieties are largely understudied. This thesis assumes that adaptation by the brown planthopper to resistant rice varieties could be due to the following which may either act alone or in combination:

1) Changes in yeast-like symbiont density mediate planthopper adaptation to resistant rice varieties.

2) Changes in the density of yeast-like symbionts facilitate host plant switching in planthoppers.

3) Virulence is acquired indirectly through horizontal transmission of virulence-promoting factors between insects feeding on the same plant (Virulence Acquisition Hypothesis).

4) Planthoppers selected on resistant varieties have increased fitness on other resistant varieties with the same or closely related resistance genes.

5) Planthoppers adapted to resistant varieties are subject to fitness costs that reduce their ability to feed on unrelated or dissimilar varieties.

Research objectives and chapter overview

This thesis aims to investigate *N. lugens* adaptation to rice varieties carrying resistance genes. The research is largely designed around a brown planthopper selection experiment with subsequent experiments linking the selection process with the density of endosymbionts as a potential component of *N. lugens* adaptation. The thesis also examines the nature of adaptation as regards shifts in the behavior of planthoppers and their ability to feed on a broader range of host varieties.

Chapter 2 recognizes that adaptation by hoppers to resistant rice varieties has been phenomenally rapid, and hopper populations with virulence against several resistance genes are now widespread. In a comprehensive review, the chapter examines the nature of the fungal and bacterial symbionts of plant- and leafhoppers and their potential function in mediating hopper virulence on rice.

Chapter 3 examines the hypothesis that increases in Yeast-Like Symbiont (YLS) density promotes planthopper adaptation to resistant varieties. A long-term selection study (20 generations of continuous rearing, ca. 24 months) was conducted with *N. lugens* on several resistant rice varieties. Planthopper fitness and the densities of YLS were monitored throughout the selection process. Based on previous studies, the chapter predicted that initially YLS densities would decline following exposure to resistant varieties when moved from a susceptible natal variety, but that the YLS densities would later increase in abundance as planthoppers adapted to resistance.

Chapter 4 examines the hypothesis that YLS density has a function in the ability of *N. lugens* to switch feeding between different rice varieties. As suggested in a previous study (Chen et al. 2011), symbionts may become a drain to planthoppers after successive generations of selection on the same host. In this chapter, adapted *N. lugens* were subjected to heat treatment that kills the majority of their YLS and generates aposymbiotic planthoppers. The fitness of aposymbiotic planthoppers was then compared to that of symbiotic (non heat-treated) planthoppers on the natal rice variety and when switched to a range of other rice varieties. If the symbionts had become a drain to the planthoppers, it was expected that aposymbiotic hoppers perform better on the rice varieties than the symbiotic planthoppers. Furthermore, if YLS were somehow adapted to a specific rice variety, then the comparative fitness of planthoppers from the different varieties was expected to vary.

Chapter 5 addresses observations from previous studies on bacterial 'contamination' in the salivary glands and feeding sites of planthoppers (Wang et al. 2008; Tang et al. 2010). The chapter examines the hypothesis (Virulence Acquisition Hypothesis) that virulence may be acquired indirectly through some unknown virulence factors that are passed between insects that contemporaneously feed on the same plant. In the study, avirulent planthoppers

are introduced to plants that were previously attacked by virulent planthoppers. It was predicted that avirulent planthoppers would gain the ability to feed and have increased fitness on resistant rice varieties when they acquired virulence factors that circulated in the plant phloem as contaminants left by virulent planthoppers.

Chapter 6 examines two hypotheses: a) that planthoppers adapted to resistant varieties would have increased fitness on similar resistant varieties, i.e., varieties with the same or closely related resistance genes; and b) that planthoppers adapted to resistant varieties would experience fitness costs that reduce their ability to feed on unrelated or dissimilar varieties. We tested the two hypotheses by examining the relative fitness of colonies that had been selected for more than 20 generations on resistant varieties when these were allowed to feed or oviposit on a range of differentials with varying levels of resistance.

Chapter 7 places the information and observations generated from Chapters 2 to 6 into the context of symbiont-mediated virulence adaptation to resistant rice varieties in *N. lugens*. The chapter discusses gaps that have been filled by the thesis research and describes future research needs.

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CHAPTER 2

Symbiont-mediated adaptation by planthoppers and leafhoppers to resistant rice varieties

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Abstract

For over 50 years host plant resistance has been the principal focus of public research to reduce planthopper and leafhopper damage to rice in Asia. Several resistance genes have been identified from native and wild rice species, and some of these have been incorporated into high-yielding rice varieties through conventional breeding. However, adaptation by hoppers to resistant rice has been phenomenally rapid, and hopper populations with virulence against several resistance genes are now widespread. Directional genetic selection for virulent hoppers seems unlikely given the rapid pace of adaptation reported from field and laboratory studies. Among the alternative explanations for rapid hopper adaptation are changes (genetic, epigenetic or community structure) in the endosymbiont communities that become advantageous for planthoppers and leafhoppers that feed on resistant rice varieties. This review examines the nature of these symbiont communities and their functions in planthoppers and leafhoppers –focusing on their likely roles in mediating adaptation to plant resistance. Evidence from a small number of experimental studies suggests that bacterial and eukaryotic (including yeast-like) symbionts can determine or mediate hopper virulence on rice plants and that symbiont functions can change over successive generations of selection on both resistant and susceptible plants. This review highlights the potential complexity of rice-hopper-symbiont interactions and calls for a more careful choice of research materials and methods to help reduce this complexity. Finally, the consequences of symbiont-mediated virulence adaptation for future rice breeding programs are discussed.

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1.0 Introduction

Since the beginning of the Green Revolution, a small group of planthoppers (Delphacidae) and leafhoppers (Cicindelidae) have continued to cause major losses to rice production throughout South and East Asia. These 'hoppers' include the brown planthopper, *Nilaparvata lugens* (Stål), whitebacked planthopper, *Sogatella furcifera* (Horvath), small brown planthopper, *Laodelphax striatellus* (Fallen), green rice leafhopper, *Nephotettix cincticeps* Uhler, and green leafhopper, *Nephotettix virescens* (Distant)(Fujita et al. 2013)(**Table 1**). Losses due to Asian planthoppers and leafhoppers have been estimated at millions of tonnes of production in some years, particularly in China, Thailand and Vietnam. Furthermore, sharp increases in the frequency and extent of hopper outbreaks have been noted since about 2002 (Fujita et al. 2013). Damage (often called 'mechanical damage' as opposed to viral symptoms) results from hopper feeding, whereby the hoppers extract nutrients directly from the rice phloem by means of a specialized stylet directed through a salivary sheath (Wang et al. 2008). In the process, large quantities of honeydew are produced on which fungal colonies can develop, often resulting in a sooty appearance of the rice (Bottrell and Schoenly, 2012; Fujita et al. 2013)(**Table 1**). Severe infestations by leafhoppers and planthoppers can lead to 'hopperburn', a wound response that results in eventual desiccation and death of the rice plant; however, even at low densities, hoppers can cause significant yield loss when they transmit rice viruses, including tungro viruses and others that cause yellowing syndromes (Fujita et al. 2013). In the Neotropics, the rice delphacid, *Tagosodes orizicolus* (Motschulsky) is a vector of Hoja Blanca Virus (HBV), a damaging rice virus that causes major losses to rice yield each year (Zeigler et al. 1994).

Table 1. Leafhopper and planthopper pests of rice.

Common name	Abbreviation	Scientific name	Host plant	Resistance genes identified from rice [1]	Reports of virulence adaptation in field populations of target hoppers [b]
Brown planthopper	BPH	*Nilaparvata lugens* (Stål)	Rice	36	*Bph1*&*bph2* – widespread virulence in S & SE Asia [1]; *Bph3*&*bph4* – India [2], Thailand [3], *bph4*&*bph8* – China and Japan [4];*BPH25*&*BPH26* – widespread in SE Asia [5];
Brown planthopper (from Leersia)[c] [6,7]	None	*Nilaparvata* sp.	*Leersia hexandra*	None	Not a rice pest
Small brown planthopper	SBPH	*Laodelphax striatellus* (Fallen)	Rice, wheat, barley, maize, oats, sugarcane, millets and some grasses	1	Unknown
Whitebacked planthopper	WBPH	*Sogatella furcifera* (Horvath)	Rice, millets, maize and some grasses	14	*Wbph1*, *Wbph2*, *Wbph3*&*wbph4* – China&Japan [4,5,8]
Rice delphacid	None	*Tagasodes orizicolus* (Muir)	Rice and weeds	None (but several rice varieties are resistant)	Unknown
Green leafhopper	GLH	*Nephotettix virescens* (Distant)	Rice and weeds	14	*Glh1* – Philippines [pers. obvs.]
Green rice leafhopper	GRH	*Nephotettix cincticeps* Uhler	Rice and weeds	7	*Grh1*&*Grh2* [9]
Zigzag leafhopper	ZZH	*Recilia dorsalis* (Motschulsky)	Rice and weeds	3	Unknown

Source references : 1, Fujita et al. (2013); 2, Verma et al. (1979) ; 3, Thanysiriwat et al. (2009); 4, Myint et al. (2009a); 5, Myint et al. (2009b); 6, Latif et al. (2008); 7, Latif et al. (2012); 8, Tanaka and Matsumura (2000); 9, Hirae et al. (2007);

[b] Gene loci names beginning with upper case letters indicate dominant genes, lower case indicates recessive genes, *BPH25* and *BPH26* were named using a more recent naming convention (Fujita et al. 2013).

[c]Recent molecular studies have indicate that brown planthopper populations feeding on rice and feeding on the weed *Leersia hexandra* represent distinct species (sibling species or cryptic species).

For over 50 years, host plant resistance has been a major focus of research for the management of leafhoppers and planthoppers. Several rice varieties and wild rice species have noticeable resistance to hoppers and many of these have been used in rice breeding programs as the donor parents of resistant rice varieties. A recent review has listed 79 resistance gene loci derived mainly from traditional rice varieties of South Asian origin (India, Bangladesh and Sri Lanka) and from a range of wild rice species (Fujita et al. 2013). Most of these gene loci were discovered only in the last 20 years; however, there is now an increasing body of evidence to indicate that many of the genes have already become ineffective in large parts of Asia because of widespread hopper adaptation (Myint et al. 2009a, 2009b; Peñalver Cruz et al. 2011; Fujita et al. 2013). It appears that there is a growing, and perhaps general virulence of hoppers to resistance genes (whereby the hoppers have adapted to feed on a wide range of resistant varieties some of which have never been extensively planted). For example, a study conducted in 2012 has indicated that 37 of 39 differential varieties, that represented donor sources for 18 planthopper resistance genes, are currently ineffective against planthoppers in the southern Indian state of Andra Pradesh (JS Bentur, personal communication). **Table 1** indicates some of the known virulent hopper populations and the genes to which they have adapted.

The pace of hopper adaptation to resistant rice varieties (which we refer to as virulence adaptation) has been phenomenally rapid. In selection studies, where hoppers are maintained in cages and fed continually on resistant rice varieties, adaptation can occur in a few generations. For example, green leafhopper adaptation to rice with *Grh2* and *Grh4* genes occurs in as little as 5 generations (Vu et al. 2014), whereas adaptation of the brown planthopper to rice varieties with *Bph1* or *Bph3* occurs at between 15 to 20 generations (Alam and Cohen, 1998). Adaptation can be partial (i.e., where the hoppers adapt to feed on a resistant variety, but lay few eggs on the same variety: Vu et al. 2014) or complete (where

hopper responses to the resistant variety become indistinguishable from those on standard susceptible varieties: Alam and Cohen, 1998; Peñalver Cruz et al. 2011). Adaptation is sometimes associated with noted behavioural changes (i.e., brown planthoppers adapted to PTB33 tend to feed higher up the plant than normal: Horgan personal observation), but more typically, behaviours are not apparently different from those on standard susceptible varieties. Under field conditions, adaptation can be similarly rapid. For example, widespread adaptation by the brown planthopper to rice varieties with the *Bph1* and *bph2* genes occurred within 5 years from first release of the varieties. These two genes, *Bph1* and *bph2*, which were extremely effective during the early years of the Green Revolution are now ineffective throughout Asia (Peñalver Cruz et al. 2011; Fujita et al. 2013). The rapid pace of adaptation suggests that virulence is unlikely to be the result of genetically based directional selection (Chen, 2009; Chen et al. 2011), challenging the 'gene-for-gene' (rice plant-for-hopper) paradigm that underlies much of our understanding of host plant resistance and pointing to other mechanisms that might determine resistance and contribute to virulence adaptations. Over the last 10 years, it has become increasingly apparent that possible alternative mechanisms underlying the rapid adaptation of planthoppers and leafhoppers to resistant rice varieties may be related to the presence and functions of the endosymbiotic gut flora that are present in all hopper species (Lu et al. 2004; Wang et al. 2010; Chen, 2009; Chen et al. 2011).

It has become increasingly clear, from a range of plant-insect associations, that symbionts mediate insect-plant interactions in multitrophic systems (Barbosa et al. 1991; Dicke, 1996; Schoonhoven et al. 2005; Frago et al. 2012). The biological phenomenon where insects live together intimately with microorganisms is called symbiosis. In an evolutionary context as pertinent to understanding insect virulence, symbiosis is not limited to the individual organisms but applies to populations. Symbiosis is ubiquitous in nature and has

been documented in at least 9 orders of insects (Hou, 2008). About 10-15% of insects are thought to harbour microorganisms that reside either extra- or intracellulary in the insect body (Douglas, 1989). Symbionts are a component of the gut flora of both planthoppers and leafhoppers (Nasu, 1963; Noda, 1974; Chen et al. 1981a, 1981b; Noda et al. 1995; Xet-Mull et al. 2004; Tang et al. 2010 ; Noda et al. 2012). Furthermore, recent studies have linked endosymbionts of the brown planthopper to variations in the outcome of rice-planthopper interactions (Lu et al. 2004; Chen et al. 2011). Several hypotheses might explain symbiont-mediated virulence. These include: (1) that hopper adaptation is determined by changes in symbiont function through shifts in symbiont community structure (abundance and taxonomic composition) over time; (2) that adaptation is related to direct genetically-based selection of symbionts that support hopper feeding (potentially gene-for-gene) – these appear rapid in the planthoppers, but constitute several multiples of generations for endosymbionts; or (3) that epigenetic shifts in symbiont gene regulation alter symbiont function and permit gradual improvements in hopper feeding over time. These mechanisms are not exclusive and may include interactions with epigenetic or genetic shifts in the hoppers themselves. **Figure 1** examines some of the likely mechanisms by which symbionts may mediate virulence adaptation in rice planthoppers and leafhoppers. It should be noted that mechanisms related to symbionts can represent the outcome of any or a combination of the above hypothesized changes in symbionts over time (i.e., during hopper adaptation).

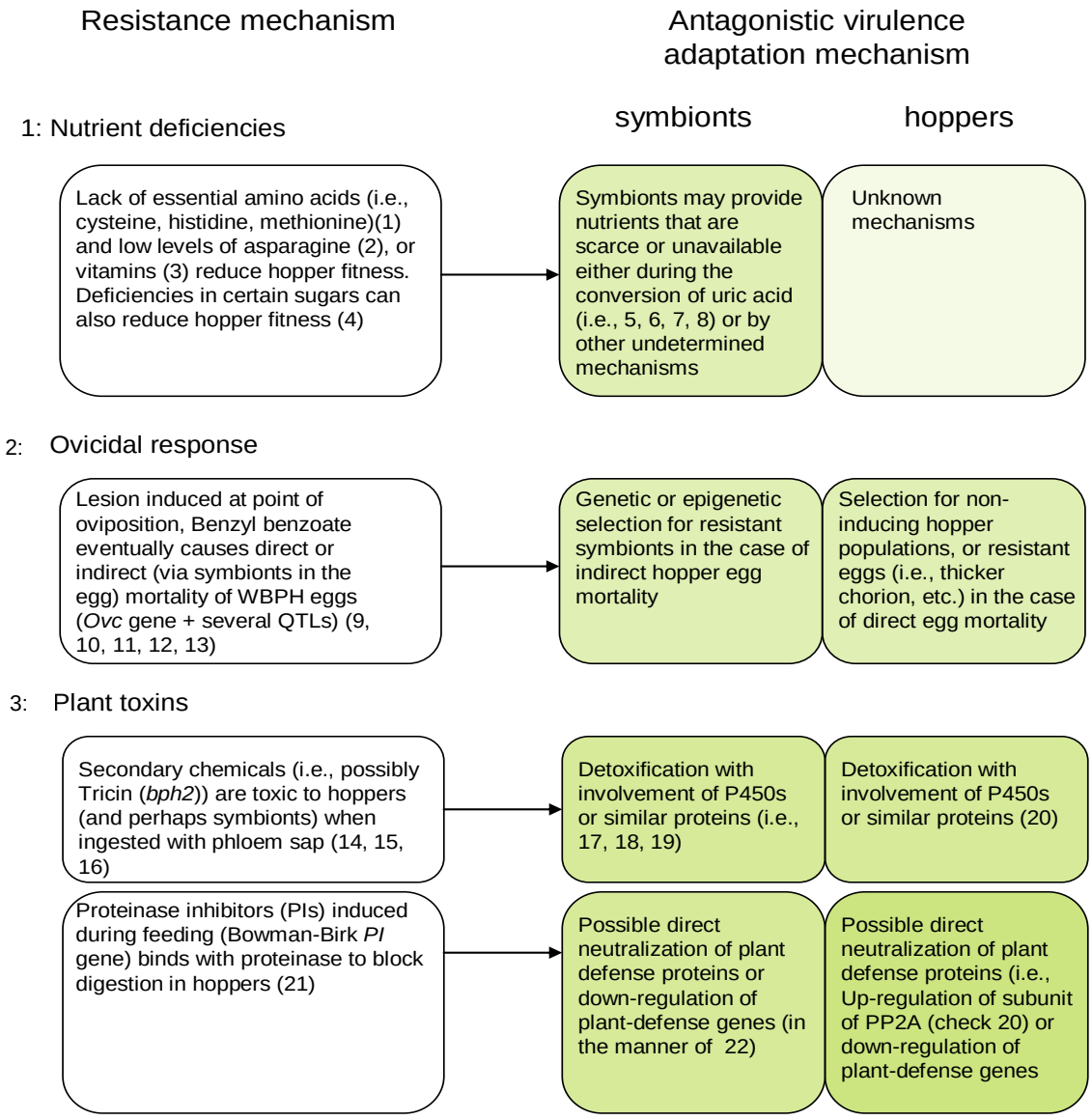
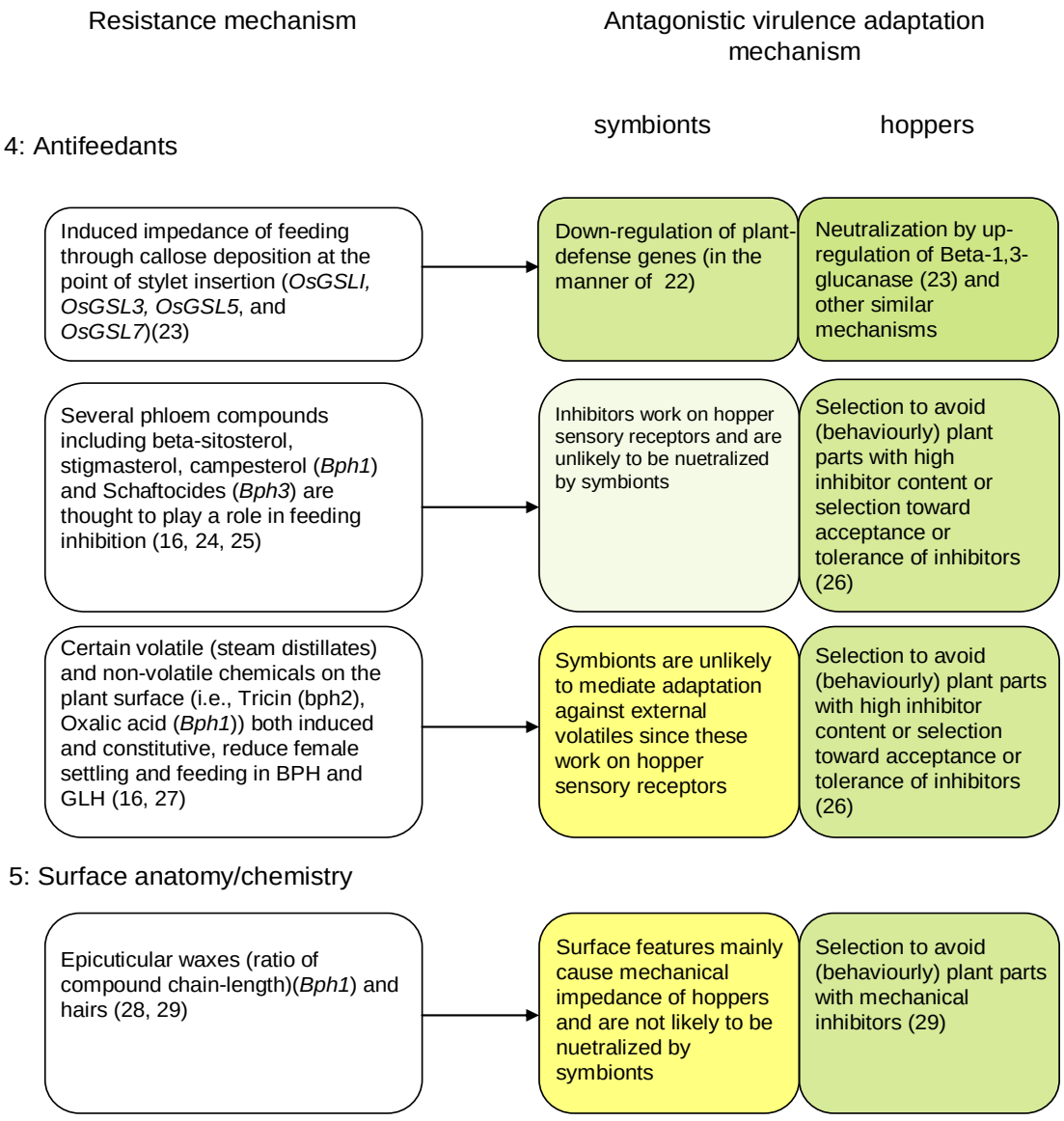


Figure 1: Rice resistance mechanisms and possible mechanisms of virulence adaptation as mediated through leafhoppers and planthoppers (the homopterans) and their symbionts. The order of resistance mechanism indicates the proposed likelihood of symbiont involvement in virulence adaptation, dark green, light green, and yellow boxes indicate high, low and zero probability of involvement by either the symbionts or hoppers in virulence adaptation. Numbers in parentheses indicate source references as follows: 1, Koyama (1986); 2, Sogawa and Pathak (1970); 3, Pathak and Kalode (1980); 4, Koyama (1985); 5, Sasaki et al. (1996); 6, Hongoh and Ishikawa (1997); 7, Ishikawa (2003); 8, Ganter (2006); 9, Sogawa (1991); 10, Suzuki et al. (1996); 11, Seino et al. (1996); 12, Kiyonaga et al. (1997); 13, Yamasaki et al. (2000); 14, Saxena and Okech (1985); 15, Yang et al. (2006); 16, Bing et al. (2007); 17, Karban and Agrawal (2002); 18, Jones (1984); 19, Dowd (1992); 20, Yang et al. (2006); 21, Weng et al. (2003); 22, Barr et al. (2010); 23, Hao et al. (2008); 24, Shigematsu et al. (1982); 25, Stevenson et al. (1996); 26, Fujita et al. (2013); 27, Yoshihara et al. (1980); 28, Woodhead and Padgham (1988); 29, Zhang et al. (2004).

Figure 1. Cont.



The purpose of this review is to collate all current knowledge on the endosymbionts of planthoppers and leafhoppers that feed on rice, highlighting the nature and function of the endosymbiotic communities present in the hopper bodies and relating these to possible shifts in hopper virulence. We examine the inoculation and population cycles of the symbionts (particularly the yeast-like symbionts) and relates these to hopper development, reproduction and behaviour. We also review known mechanisms of rice resistance and examine probable roles for endosymbionts during hopper virulence adaptation. In particular, we critically examine available experimental evidence that draws links between endosymbionts and virulence adaptation and discuss possible directions for future research in this area. Finally, we highlight major gaps in the current knowledge, discuss the application of novel entomological, microbiological and molecular tools in elucidating complex interactions, and discuss some possible consequences from what is known of symbiont-hopper-rice interactions for the successful breeding and deployment of resistant rice varieties.

2.0 Planthopper-symbiont associations

Nasu (1963) was the first to report symbionts associated with rice planthoppers. Until the early 2000s, yeast-like symbionts (YLS) had been the only type of symbiont found in planthopper species (Noda, 1974; Chen et al.1981a; Kagayama et al.1993; Noda et al. 1995; Espinosa et al. 2004; Xet-Mull et al. 2004). A number of studies have established roles for these YLS in planthopper nutrition, reproduction and development (Noda et al.1979; Koyama, 1985; Lee and Hou, 1987; Eya et al.1989; Sasaki et al. 1996; Hongoh and Ishikawa, 1997; Wilkinson and Ishikawa, 2001; Noda and Koizumi, 2003). More recently bacterial symbionts were discovered associated with the brown planthopper (Tang et al. 2010): bacteria-like organisms have also been observed in the salivary sheets of the brown planthopper using electron microscopy (Wang et al. 2008a; Tang et al. 2010). The role of bacterial endosymbionts in hoppers has not been elucidated, although there is some evidence

that these are involved in nutrition and may also participate in determining planthopper virulence (Wang et al. 2010). Recently, the presence of symbionts has also been confirmed in rice leafhoppers (Noda et al. 2012). Histological studies had identified two bacteriome-associated symbionts and a rickettsial microorganism in the green rice leafhopper (Nasu, 1965; Mitsuhashi and Kono, 1975) but these were not identified until very recently (Noda et al. 2012). Cloning and sequencing of the 16S ribosomal RNA gene generated a major sequence which was placed in the Bacteroidetes clade of *Sulcia muelleri*, a symbiont lineage associated with various hemipteran insects; another major sequence was related to a β-proteobacterial sequence from a leafhopper *Matsumuratettix hiroglyphicus* while a minor sequence was grouped in the α-proteobacterial genus *Rickettsia* (Noda et al. 2012).

2.1 Taxonomy and phylogeny of planthopper symbionts

The primary (obligate) symbionts of planthopper and leafhopper cannot be cultured in vitro (Noda et al. 1995). For this reason research into hopper symbionts has remained relatively understudied when compared to, for example, aphid symbiosis – where many of the symbionts may be secondary. Nevertheless, symbionts can be isolated from planthopper and leafhopper tissues through density-gradient ultracentrifugation (Noda and Omura, 1992), which has permitted research into their taxonomic affiliations without the need for the pure culture isolates required with most conventional methods of microorganism classification (Ganter, 2006). In addition, sequence information of nucleic acids or proteins can be useful for the study of these obligate symbionts because of the difficulties in growing the symbionts in vitro (Noda et al. 1995). Information on the relationships between different YLS can help in determining the phylogenetic origin and microbial affiliations of these symbionts (Noda et al. 1995) and could provide information on how herbivores acquired symbionts and on the evolution of herbivory (Clark et al. 2010).

Several different symbiotic microorganisms occur in planthoppers and leafhoppers (**Table 2**). Using primer sequences designed to amplify the conserved 18S ribosomal DNA region (rDNA), Noda et al. (1995) located YLS in the class Pyrenomycetes, subphylum Ascomycotina in the fungal kingdom and suggested that the YLS from 3 rice planthoppers: The brown planthopper, the whitebacked planthopper and the small brown planthopper were monophyletic i.e., derived from a single ancestral species. YLS have also been isolated from the rice delphacid (Espinosa et al. 2004). Recently, Dong et al. (2011) identified two types of YLS isolated from fat bodies of the brown planthopper as *Cryptococcus*- and *Pichia*-like symbionts. Isolation and identification was achieved through amplification of the 18S and ITS-5.8S rDNA sequences with universal fungal primers. Surprisingly, there was a high degree of similarity between YLS of the three Asian rice planthopper and those of the South American planthopper (98% similarity) suggesting a common Pyrenomycete ancestor among the 4 YLS despite their geographic isolation (Xet-Mull et al. 2004). Therefore, rice planthopper YLS seem to be highly conserved implying that rapid speciation events and significant genetic changes may be rare. This goes against the hypothesis that planthopper YLS may shift from time to time to provide genetic variations that will directly affect host fitness and reproductive success on different rice varieties.

In recent years, researchers have begun to examine the bacterial endosymbionts of hoppers in more detail (i.e., Tang et al. 2010; Wang et al. 2010; Noda et al. 2012). Eighteen bacterial operational taxonomic units (OTUs) which represent four phyla have been identified from the brown planthopper. These uncultured bacteria were detected by extracting the total genomic DNA of the planthopper and amplifying the 16S rRNA gene. The OTUs belonged to four different phyla - Proteobacteria (13 OTUs), Firmicutes (2), Actinobacteria (2), and Bacteroides (1). Comparisons of the 16S rRNA sequences of these OTUs indicated a similarity between planthopper bacterial symbionts and the secondary symbionts or gut-

associated microbes of other insect species, although some planthopper symbiont OTUs had not previously been found in insects (Tang et al. 2010). In contrast to YLS, the study of Tang et al. (2010) suggested that the identities (i.e., species) and numbers of bacterial OTUs differed substantially between brown planthoppers reared on different rice varieties (TN1, a standard susceptible variety, Mudgo, which possesses the *Bph1* gene, and ASD7, which possesses the *bph2* gene) (Tang et al. 2010). Unfortunately, in their study, Tang et al. (2010) did not replicate their selected hopper colonies (see below), and many of the observed bacterial OTUs may represent secondary symbionts that are not required for hopper survival. Nevertheless, their study combined with observations on the similar taxonomic origins of YLS (Xet-Mull et al. 2004), suggests that shifts in the community composition of bacterial symbionts, but not in YLS symbionts, may play a role in determining hopper fitness on rice varieties and could therefore determine or assist in virulence adaptation.

2.2 Distribution, location and transmission of symbionts in hoppers

Symbionts can live extracellularly, i.e. in the gut lumen or digestive tract of insects, or intracellularly, i.e., inside a specialized cell type (mycetocyte) in the insect (Douglas, 1989: **Table 2**). Mycetocyte symbionts are beneficial to the insects that contain them: When these microbes are eliminated, the insects grow and develop slowly and die prematurely, often without reproducing (Douglas, 1989).

Table 2. Eukaryotic and prokaryotic microorganisms associated with rice leafhoppers and planthoppers; some studies may have reported mutualistic symbionts as well as yeast-like and bacterial contaminants identified during screening.

Leafhopper and planthopper species	Insect developmental stage	Symbionts identified	Symbiont location	Symbiont function
Brown planthopper	Eggs, nymphs, adults [1,2]	Yeast-like (Pyrenomycete-Ascomycotina) [3], *Cryptococcus*- and *Pichia*-like symbiotes [4]; bacteria (*Proteobacteria, Firmicutes, Actinobacteria, Bacteroidetes, Candida)* [5]	abdominal fat body; ovary [1]	Nitrogen waste recycling [6,7]; supply proteins for embryonic development [8]; provision of amino acids for nymphal development; sterol provision [9]
Smaller brown planthopper	egg, nymph, female adult and male adult [10]	Yeast-like (Pyrenomycete-Ascomycotina) [3]	abdominal fat body; ovary [10, 11]	Source of sterols for host development[9]
Whitebacked planthopper	Eggs, nymphs, adults [11]	Yeast-like (Pyrenomycete-Ascomycotina)[3]	abdominal fat body; ovary [11]	Unknown
Rice delphacid	Eggs, nymphs, adult [12]	Yeast-like (Pyrenomycete-Ascomycotina) [13]	abdominal fat body; ovary [12]	Unknown
Green rice leafhopper	Nymphs, adults [14]	Bacteria (*Sulcia* bacterium and *β*-proteobacterium; Rickettsia)[14]	Bacteriome; ovary bacteriome, ovary, testis, midgut, Malpighian tubules, fat body [14]	Unknown

1, Chen et al. (1981b); 2, Nasu (1963); 3, Noda et al. (1995); 4, Dong et al. (2011); 5, Tang et al. (2010) ; 6, Sasaki et al. (1996) ; 7, Hongoh and Ishikawa (1997); 8, Lee and Hou (1987) ; 9, Eya et al. (1989) ; 10, Noda, (1974) ; 11, Noda, (1977) ; 12, Espinosa et al. (2004); 13, Xet-Mull et al. (2004) ; 14, Noda et al. (2012).

This association is specifically called mycetocyte symbiosis and is characterized by the following conditions: the microorganisms are intracellular and restricted to the cytoplasm of the special insect cell type - the mycetocyte; the microorganisms are maternally inherited; the association is required by both the insect and microbial partners (Douglas, 1998). The location of the mycetocytes differs between insect groups. Mycetocytes are usually found in the cells of the digestive tract, in the abdominal haemocoel or in the fat body of the abdomen (Douglas, 1989). In the small brown planthopper, mycetocytes have been found only in the fat body cells of the abdomen and not in other parts of the insect body (Noda, 1974).

Cycles in the occurrence and abundance of YLS were first described for the small brown planthopper (Noda, 1974): YLS are present at every developmental stage of the hopper (**Figure 2**) with the number of symbionts increasing from egg to adult stage. According to a detailed study by Noda (1974), the abundance of YLS in the small brown planthopper peaks at 8-10 days after adult emergence in both brachypterous (short-winged) and macropterous (long winged) females, but numbers are lower in the latter. By the fifth instar, there are substantially more symbionts in female nymphs than in males. Furthermore, the number of symbionts drops sharply in males following emergence of both brachypterous and macropterous individuals (**Figure 2**). This suggests that high densities of YLS in adult females may be associated with egg production, especially since brachypterous females (adapted for reproduction) produce more eggs than macropterous females (adapted for dispersal) (Padgham, 1983). Similar trends have been found with YLS of the brown planthopper (Chen et al. 1981b; Cheng and Hou, 2001; Lu et al. 2004; Hou, 2008).

In the Homoptera, symbionts are passed from parents to progeny through the egg. This ‘transovarial transmission’ of YLS has been extensively documented in rice planthoppers (Noda, 1977; Lee and Hou, 1987; Nagamine et al. 1993; Kagayama et al. 1993; Cheng and Hou, 1996; Cheng and Hou, 2001). The mechanism of YLS transmission from the

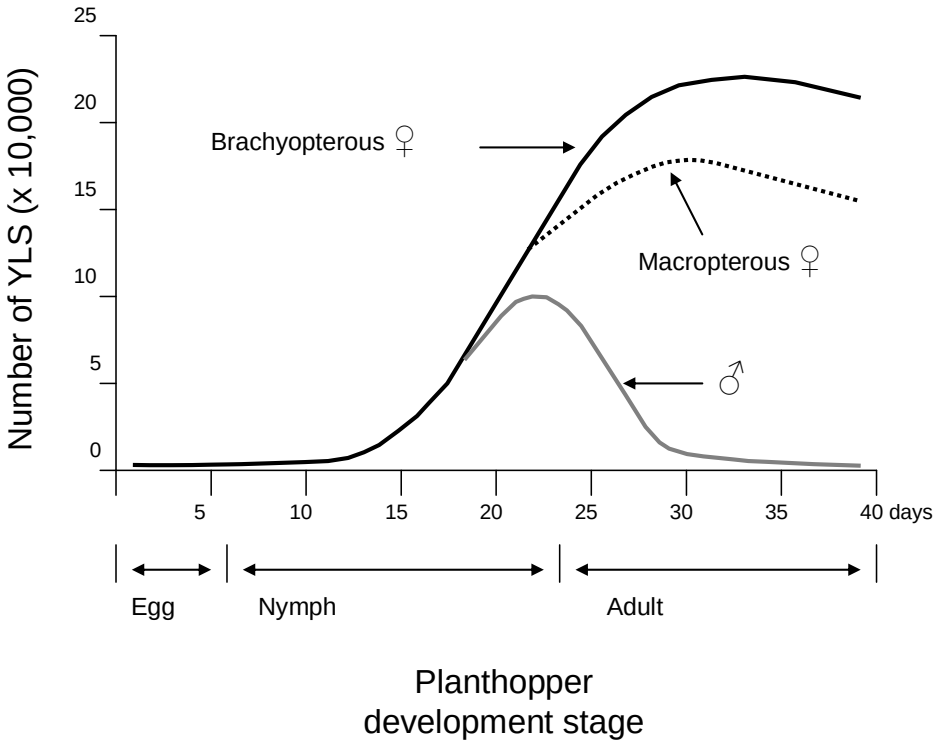


Figure 2. Changes in the number of yeast-like symbionts (YLS) during the life cycle of the small brown planthopper, *Laodelphax striatellus* (redrawn from Noda, 1974).

fat body to the oocyte was revealed by both light and electron microscopy and has been divided into a series of recognizable stages (Cheng in Hou, 2001): (1) the YLS in the mycetocytes first move out of the syncytium (formed from a layer of fat body cells) by exocytosis, and are released into the hemocoel; (2) the free YLS in the hemolymph then move to the ovarioles near the pedicel which is enclosed by follicle cells; (3) the YLS then enter the follicle cells around the primary oocyte by endocytosis at the epithelial plug of the ovariole; (4) finally, the YLS assemble at the posterior end of the mature egg forming a symbiont ball (Cheng and Hou, 2001). Therefore, YLS are maternally transmitted from generation to generation (Lee and Hou, 1987; Cheng and Hou, 2001). The intricacy of the mechanisms ensuring transovarial transmission of YLS have indicated that symbionts are involved in essential, beneficial functions for the insect host (Houk and Griffiths, 1980) and particularly in reproduction and embryonic development (Lee and Hou, 1987; Cheng and

Hou, 2001). The mechanisms behind the transmission of bacterial symbionts from one generation to the next in planthoppers have not yet been elucidated, although some bacterial symbionts are found in the ovaries (**Table 2**). Whether this transmission is horizontal or vertical could have profound consequences for planthopper and leafhopper virulence adaptation.

3.0 The Role of Symbionts in Rice Planthoppers

Endosymbiotic microorganisms can play a role as mediators that promote feeding and development of herbivores on their host plants by improving plant suitability for the herbivore and/or by improving the herbivores ability to utilize the plant (Krischik and Jones, 1991; Dicke, 1996; Schoonhoven et al. 2005; Frago et al. 2012). Historically, research into insect-microbe associations was motivated by the observation that symbiont-bearing insects usually feed on diets that are nutritionally insufficient (Richards and Brooks, 1958). Therefore, the acquisition of symbionts by insects can be considered a key innovation in the evolution of herbivory (Moran and Telang, 1998) that has allowed insect adaptation to unexploited ecological niches that are nutritionally poor and/or unbalanced (Moran et al. 2003; Chaves et al. 2009).

3.1 Nutrition and development

The relationship between symbionts and insects is mainly nutritionally-based. The microorganisms associated with insects provide nutrients that are scarce or unavailable in the insect diet and the symbiotic microorganism in return gains a permanent supply of several metabolites produced by the insect host (Chaves et al. 2009). Plant phloem sap is the primary diet of most homopterans, including planthoppers and leafhoppers. This food source is rich in carbohydrates but poor in essential amino acids. Hoppers feeding on resistant rice varieties are thought to be further deprived of essential nutrients, including sugars, amino acids, and possibly vitamins (Sogawa and Pathak, 1970; Pathak and Kalode, 1980; Koyama, 1985,

1986; Jung and Im, 2005; Chen et al. 2011) and their bodies have reduced uric acid and fat (including crude fat) content (Yin et al. 2008; Hongoh and Ishikawa, 1997; Sasaki et al. 1996) and reduced lipid synthesis and glycogen reserves (Padgham, 1983). The role of endosymbionts (bacteria and YLS) is seemingly to supply essential amino acids that are lacking in the insect diet (Wilkinson and Ishikawa, 2001; Ganter, 2006). In resistant rice, poor nutritional quality of the host can be directly due to deficiencies in the phloem or can arise due to the inability of hoppers to access the nutrients present in the phloem (either as a result of antifeedants or other plant defense mechanisms). For example, in a study by Jung and Im (2005), brown planthoppers feeding on the resistant variety Cheongcheongbyeo excreted significantly less sugars than hoppers feeding on a susceptible variety, despite similar sugar contents in the phloem of both varieties. This suggests that unidentified phloem components can disturb or alter planthopper digestive or feeding processes on resistant varieties. The amino acid composition of rice phloem is known to differ markedly between rice varieties (see Chen et al. 2011 and references therein). Using artificial diets, the absence of sulphur-containing amino acids (cysteine, histidine and methionine) has been shown to negatively affect planthopper fitness (Koyama, 1986) although the absence of any one amino acid appears not to affect the hoppers. Whether symbionts could eventually neutralize the effects of dietary deficiencies or compensate for low concentrations of key amino acids is still unknown; however, it is intuitive that dietary compensation mediated by endosymbiotic gut flora (particularly YLS) will underlie adaptation by planthoppers to certain resistant rice varieties (**Figure 1**).

The nutritional role of YLS has been studied extensively in the planthoppers and is known to contribute to the nitrogen requirements of these insects (Douglas, 1989). Planthoppers produce uric acid as a nitrogenous waste but do not excrete it as occurs in many other insects. For the brown planthopper under high nitrogen diets, uric acid is stored in the

insect tissues and converted into compounds of nutritional value by YLS through the action of symbiont uricase when the hoppers are under nitrogen stress (Sasaki et al. 1996; Hongoh and Ishikawa, 1997; Ishikawa, 2003; Ganter, 2006). Interestingly, the levels of uric acid in the brown planthopper egg are highest at oviposition and decrease significantly as the egg develops. This suggests that the egg is supplied with uric acid by the parent prior to oviposition at about the time that YLS are most abundant in the female's body (Hongoh and Ishikawa, 1997).

The role of endosymbionts becomes most apparent when these are removed from the hopper body. For example, submitting hoppers to heat treatment can reduce YLS numbers significantly, and this has become a standard in studies of hopper symbionts. Aposymbiotic planthoppers produced by heat treatment demonstrate several physiological and developmental deficiencies. Vega and Dowd (2005) have summarized the effects of heat-reduction of YLS in brown planthopper eggs and nymphs. They listed the following observations drawn from a number of different studies: (1) in the egg, normal embryonic and postembryonic development are affected due to the absence of certain proteins synthesized by YLS (Lee and Hou, 1987); (2) there is a reduction in egg hatching and (3) an increase in the duration of each nymphal stage (Bae et al. 1997; Zhongxian et al. 2001); (4) there is failure to moult resulting in the death of fifth instars during ecdysis (Chen et al. 1981a); and finally, (5) insect weight, growth rate and the amount of protein per unit of fresh weight are reduced (Wilkinson and Ishikawa, 2001). In contrast to the severe effects on eggs and nymphs, when YLS are removed/reduced from adult planthoppers by heat treatment there are no effects on mortality or lifespan, suggesting that YLS are not directly involved in adult survival (Lee and Hou, 1987). However, YLS do play a role in determining the fertility of planthoppers since heat-treated females lay fewer eggs (Ganter, 2006).

Evidence of sterol synthesis by YLS has been demonstrated through comparisons of control and heat-treated individuals of the small brown planthopper: Heat treatment resulted in failure of fifth instars to moult to adults, leading Noda and Saito (1979) to conclude that YLS are involved in sterol metabolism. Further investigation by Noda et al. (1979) suggested that YLS were responsible for the production of 24-methylenecholesterol and that the concentration of this cholesterol was significantly reduced in heat-treated insects where the YLS had been destroyed (or significantly depleted). Twenty-four methylenecholesterol can be synthesized by YLS maintained in culture broth isolated from eggs of the brown and small brown planthoppers (Eya et al. 1989). Several other sterols such as trienol 6 (major sterol found), cholesterol and lanosterol 8 have also been found in YLS. These were isolated through density gradient centrifugation from the brown and small brown planthoppers (Wetzel et al. 1992). Clearly YLS are involved in the nutrition of planthoppers, and, although studies have so far not been conducted, they are also likely to play a role in leafhopper nutrition. The ability of YLS to alter/process food inputs to provide essential nutrients for hoppers suggests that the symbionts play a key role in determining host choice and virulence adaptation. Furthermore, YLS-mediated adaptation to resistant rice varieties may be more prominent in those rice varieties for which resistance is due to phloem nutrient deficiencies (i.e., poor food quality for hoppers)(**Figure 1**).

3.2 Detoxification of plant allelochemicals

Some resistant rice varieties produce secondary chemicals that prevent feeding by planthoppers and leafhoppers. Some of these chemicals act as antifeedants (i.e., C-glycosidic flavonoids in varieties with the *Bph3* gene: Stevenson et al. 1996) but others are apparently toxic to the hoppers. For example, when planthoppers feed on B5 (which contains the *Bph14* and *Bph15* genes) a P450-encoding planthopper gene is activated (Yang et al. 2006). P450s are best known for their role in the metabolism of insecticides and plant secondary chemicals

– indicating that B5 produces toxic substances that are ingested by the planthoppers. When planthoppers feed on B5 or other resistant varieties, their endosymbiotic gut flora are also exposed to the plant toxins (i.e., Dowd, 1992). Enzymatic detoxification systems (such as P450s) are widespread among herbivores and some of these are provided as services by microbial symbionts (Karban and Agrawal, 2002; Jones, 1984, Dowd, 1992). Insect symbionts (e.g., bacteria and YLS) are known to play a role in the detoxification of plant toxins as well as man-made compounds such as insecticides (Barbosa et al. 1991; Kikuchi et al. 2012). Microbial-mediated transformation of plant secondary compounds (i.e., flavonoids, tannins, and alkaloids) in the insect gut has been demonstrated from a few studies indicating that microbial symbionts can determine the ability of phytophagous insects to overcome barriers to herbivory (Douglas, 1989; Dillon and Dillon, 2004).

There are no clear examples of symbiont-mediated detoxification of plant compounds in planthoppers or leafhoppers; however, if symbionts are mediating detoxification of rice toxins during hopper feeding, then the processes may be similar to those found in other symbiont-herbivore systems. One nice example of YLS detoxification of plant secondary chemicals has come from studies with *Symbiotaphrina kochii* - a YLS of the cigarette beetle, *Lasioderma serricorne* (Fabricius). A study of symbiosis between these two species has demonstrated localized detoxification activity of 1-naphthyl acetate and tannin in the yeast mycetosomes which assists the beetle in surviving on toxic dried plant substrates (Dowd, 1989). In a further study on a similar system, aposymbiotic cigarette beetles had high mortality when treated with representative plant defence toxins such as *trans*cinnamic acid, trihydroxyflavone, flavone and tannic acid, compared to control beetles with normal symbiont communities (Dowd and Shen, 1990). Furthermore, in a study to investigate whether midgut symbionts mediate detoxification of plant glycosides, saligenin, the aglycone of salicin (a plant glycoside), was added to an artificial diet fed to *Tenebrio molitor* (L.)

larvae. A group of larvae had been reared antiseptically from surface sterilized eggs and by feeding larvae with food containing antibiotics. These larvae were therefore free of gut lumen bacteria, yeasts and fungi and had lower larval weight gain and premature pupation compared to conventionally reared larvae, suggesting a detoxifying role of the midgut microbiota (Genta et al. 2006). Because several toxins (including antifeedants) are known from rice, such manipulative experiments are feasible and could help identify the role of symbionts in virulence adaptation by planthoppers and leafhoppers.

In spite of the high number of planthopper and leafhopper resistance-gene loci that have been identified in rice (Fujita et al. 2013), and the development of near isogenic rice lines with and without resistance loci (Fujita et al. 2010; Fujita et al. 2013), there is still very little information on the functioning of resistance genes in rice. Furthermore, rice plant compounds that are toxic to planthoppers and leafhoppers have not been successfully linked to any major rice resistance genes (Horgan, 2009). To complicate things further, it is often difficult to distinguish the roles of plant compounds in defence, for example the flavonoid 5,7,4'-trihydroxy-3',5'-dimethoxyflavone (tricin), which has been isolated from IR36 (*bph2* gene) reduces feeding and development in brown planthopper nymphs (Bing et al. 2007), but this could be either through direct toxic effects on the hoppers or through antifeeding mechanisms – both of which result in the same gross symptoms during hopper development (Horgan, 2009); It is thought that most of plant compounds that have so far been associated with resistant varieties and linked to decreased hopper fitness are feeding inhibitors that are non-toxic (i.e., certain sterols [Shigematsu et al. 1982], oxalic acid [Yoshihara et al. 1980] and schaftosides [Stevenson et al. 1996]). Since resistance mechanisms have only been studied for a small number of resistance genes (*Bph1*, *bph2*, *Bph3*, *bph4*, *Bph14*, *Bph15*), it is possible that toxic secondary chemicals will be identified in association with some of the many remaining resistance genes in future studies. However, in terms of a role for symbionts

in virulence adaptation, it will be important to clearly distinguish whether gene products are toxic, antifeedant, or both. Nevertheless, because microorganisms evolve faster than insects, it is likely that symbiont-mediated detoxification of plant compounds occurs during hopper exposure to resistant rice in a similar manner to bacteria-mediated detoxification of insecticides (Kikuchi et al. 2012).

3.3 Down-regulation of plant defence genes

Under normal circumstances, plant defence signalling pathways such as the salicylic acid (SA)-, jasmonic acid (JA)- and ethylene pathways are activated during interactions between plants and their attackers (pathogens and/or herbivores)(Pieterse and Dicke, 2007). Plant defence inducers such as beta-glucosidase present in the saliva of the brown planthopper have already been associated with SA, ethylene and hydrogen peroxide production (Wang et al. 2008a, 2008b). Studies have indicated that the JA-pathway may also be activated during planthopper attack (Zhang et al. 2004; Wang et al. 2008b). The induction of these phytohormones by planthoppers regulates the synthesis of feeding inhibitory (i.e., callose: Hao et al. 2008) and digestibility reducing compounds (i.e., proteinase inhibitors: Weng et al. 2003) as well as a variety of volatile organic compounds (i.e., linalool, (3E)-4,8-dimethyl-1,3,7-nonatriene, indole: Xu et al. 2002). Generally, insect herbivores employ offensive strategies to counter plant-imposed challenges: For example, planthoppers employ beta-1,3-glucanase breakdown of callose (Hao et al. 2008) and can up-regulate the B-subunit of PP2A in response to plant PPA2 production (Yang et al. 2006). It is possible that planthoppers and leafhoppers possess suites of sophisticated nuclear genes that code for these counter defences; however, it is also possible that hoppers might acquire such functional innovations through established symbiotic associations. For example, Barr et al. (2010) have shown that a symbiont, rather than the phytophagous insect itself was involved in down-regulating several genes involved in the defence of maize (*Zea mays* L.) against the western

corn rootworm, *Diabrotrica virgifera virgifera* Le Conte. Analysis of microarray expression data showed genome-wide suppression of maize defence genes (i.e., cell wall defences, production of phytoalexins and pathogenesis-related proteins) following attack by the rootworm where *Wolbachia*, an intracellular bacteria found throughout the rootworm body, was naturally present. However, when the corn rootworms were treated with antibiotics (eliminating *Wolbachia*) these same maize defence genes were up-regulated (Barr et al. 2010). It is possible that the symbionts of planthoppers are also normally involved in the down regulation of rice defences (leading to negative effects in aposymbiotic planthoppers even on seemingly susceptible rice varieties), but that the symbionts also activate counter defences when hoppers are reared continually on resistant varieties.

4.0 Implications of symbiosis for herbivore adaptation to rice plant resistance

The process of evolution involves hereditary variation which ultimately arises by mutation, followed by selection in which the most successful variants contribute more to the next generation than the less successful variants. Conventionally, the pest status of insects has been largely attributed to their genomes. However, at least one study has ascribed pest-related traits primarily to a symbiont genotype rather than the insect genotype itself: Hosokawa et al. (2007) observed that the pest stinkbug, *Megacopta punctatissima* (Montandon), which performs well on crop legumes, has a closely related non-pest conspecific, *Megacopta cribraria* (Fabricius), which performs poorly on the same plants in terms of egg hatching rate. In a manipulative experiment, the authors exchanged the obligate gut symbiotic bacteria between the two insect species and demonstrated a marked reversal in their performance on the crop legumes. *Megacopta punctatissima* with foreign symbionts had a low hatching rate on their normal legume host due to the mortality of nymphs before or upon hatching (Hosokawa et al. 2007). The results of the study clearly imply that endosymbionts influence the performance of herbivores on plants, to such an extent that they can ultimately determine

whether a plant becomes a suitable host for the insect or not. It would be interesting to conduct similar studies on, for example, the two cryptic species that constitute the brown planthopper complex (Latif et al. 2008, 2012: **Table 1**), one of which is largely monophagous on rice and the second on the grass weed *Leersia*. Similarly, because YLS are passed horizontally from generation to generation through the egg, and are therefore linked to the female parent only – simple reciprocal mating experiments between selected populations on resistant and susceptible rice lines could help determine the extent to which observed virulence is determined by symbionts. Preliminary mating studies with the brown planthopper have indicated that, whereas YLS did contribute to virulence on a resistant variety (IR62 – *Bph3* gene), the male parent also played a role, suggesting that other mechanisms (which may include bacterial symbionts) also played a role in virulence adaptation (unpublished results, Peñalver Cruz et al 2011).

The summary of known rice resistance mechanisms against planthoppers and leafhoppers presented in **Figure 1** (white boxes) indicates the diversity and complexity of these mechanisms. This diversity of resistance mechanisms, together with observations on the frequency and extent of planthopper and leafhopper adaptation to resistant rice varieties (**Table 1**), suggests that virulence adaptations are likely to be similarly diverse and complex. Virulence adaptation against specific varieties, or the products of specific resistance genes, may involve symbionts alone (either YLS, bacterial symbionts, or both), involve the hoppers alone, or involve interactions between the hoppers and their symbionts. Furthermore, adaptation could be mediated through community shifts in symbionts, genetic or epigenetic shifts in the symbionts or genetic and epigenetic shifts in the hoppers. There may also be more complex interactions that include, for example, community shifts in symbionts that are mediated through epigenetic shifts in the hoppers. Teasing the exact mechanisms apart will be a difficult task. It has also becoming increasingly apparent that major resistance rice genes

interact with other genes located throughout the rice genome to determine the extent and durability of the rice plant's resistance to hoppers (Fujita et al. 2013). This has been demonstrated nicely in studies of the ovicidal response of japonica rice to eggs of the whitebacked plant hopper (Yamasaki et al. 1999) and is demonstrated in the complex of resistance mechanisms associated with the *Bph1*-gene (see **Figure 1**). Furthermore, the same genes present in different variety backgrounds, have often strikingly distinct effects on hopper populations (sometimes ranging from susceptible to resistant, i.e., Peñalver Cruz et al. 2011), and hoppers require a few generations to attain maximum fitness even on encountering seemingly susceptible varieties (Claridge and Den Hollander 1982, 1983: Alam and Cohen, 1998, but see Chen et al. 2011). Indeed movement between any two varieties with all their complex of allelochemicals and phloem components seems to constitute a barrier for planthopper and leafhopper feeding (Horgan, 2012).

Given the existing knowledge on the role of YLS in nutrition, it is intuitive that these symbionts play a key role in mediating hopper adaptation to varieties that are resistant due to phloem nutrient deficiencies, or deficiencies caused by antifeedants that block the extraction or assimilation by the hoppers of phloem nutrients. Lu et al. (2004) have demonstrated an initial reduction in YLS numbers after brown planthoppers were switched between rice varieties (susceptible to resistant), followed by a gradual increase in symbiont numbers over successive generations of selective rearing on the resistant varieties. The activities of alanine transminase and aspartic transaminase were significantly reduced in aposymbiotic planthoppers during feeding and were different between planthopper populations selectively reared on distinct host plants, suggesting that symbionts contributed differently to amino acid utilization efficiency on the different plant hosts (Lu et al. 2004).

In a more detailed study, and using the same rice varieties, Chen et al. (2011) found changes in amino acid composition of the brown planthopper (all body structures) after

several generations of selective rearing, with total nitrogen content and the concentration of rare amino acids increasing, but some common amino acids decreasing over successive generations. These shifts in amino acid composition appeared to be influenced more by the selection process itself, rather than the host on which the hoppers were selectively reared or the host on which they had most recently fed (where hoppers were switched between hosts before analyses). Examination of the hopper colonies during two generations (the 8th and 11th) revealed that YLS improved nymphal performance in the 8th generation, but appeared to be a drain on nymphs in the 11th generation (as shown by the higher performance of the aposymbiotic nymphs than the symbiotic nymphs in the 11th generation)(Chen et al. 2011). It seems apparent therefore, that YLS do mediate adaptation to different rice varieties. In the study by Lu et al. (2004), hoppers had been reared in isolation for several generations to ensure that the host plants were resistant, whereas in the study by Chen et al. (2011), the selected colonies were already virulent against the same varieties. This might explain some of the differences between the studies and indicates that the role of symbionts in mediating virulence will differ depending on the strength of rice resistance and the extent of planthopper exposure to the resistant variety or to varieties with similar resistance genes/mechanisms. YLS may mediate virulence adaptation in early generations of selection, with the planthoppers themselves ultimately adapting to the novel resistance after several generations – the role of the YLS becoming reduced and possibly representing a cost to the insect.

In a further study on endosymbiont mediated virulence adaptation, Wang et al. (2010) found that the composition and abundance of bacterial symbiont OTUs differed among brown planthoppers populations reared on the same varieties as in the previous two studies (Wang et al. 2010). It appears therefore that both bacterial symbionts and YLS can mediate virulence adaptation. As discussed earlier, this is unlikely to involve shifts in the composition of YLS communities, but may involve changes in YLS abundance (Lu et al. 2004) and/or function

(Chen et al. 2011). Meanwhile both endosymbiotic bacterial community composition and function may shift to mediate virulence adaptation. The function of bacterial symbionts could further change through genetic or epigenetic shifts occurring over several generations of selective rearing of hoppers. Changes in bacterial function, and even the function of YLS, through genetic changes or epigenetic shifts could also mediate detoxification of plant compounds that act directly on the symbionts themselves or indirectly by affecting the hoppers (**Figure 1**). In a similar manner, symbionts might play a role in countering defensive plant proteins that act as feeding inhibitors; however, where feeding inhibitors act externally (i.e., by mechanically blocking stylets (i.e., Hao et al. 2008) or through antixenotic volatiles or surface chemicals that act on insect sensory receptors, symbionts will clearly have a reduced role in mediating virulence adaptation (**Figure 1**). Interestingly, most of the current research on planthopper and leafhopper resistance aimed at resistance gene discovery and resistance breeding in rice employs a standardized seedling test that has a bias toward antibiosis resistance mechanisms – rather than antixenosis mechanisms. Furthermore, the seedling tests are generally restricted to nymphs (Horgan, 2009; Fujita et al. 2013). This suggests that many, if not most, of the currently available resistant rice varieties are susceptible to symbiont-mediated virulence adaptation and may even target the symbionts themselves. This strongly questions the current gene-for-gene paradigm (rice genes interacting with planthopper genes) that permeates rice resistance research and suggests that genetic studies aimed at identifying hopper gene as markers for virulence might be misdirected because the gene-for-gene relationship that researchers are seeking involve rice genes interacting with the genes of endosymbiotic microorganisms instead. This might indicate that variety deployment methods designed to reduce the rate of pathogen adaptation (Leung et al. 2003) might be equally valid in managing hopper resistant rice varieties.

In contrast to the more common antibiosis feeding-resistance in rice (described above), ovicidal resistance targets the eggs of planthoppers (mainly the whitebacked planthopper, but also the brown planthopper). The ovicidal response was first identified by Sogawa (1991) when he noted dark brown discoloration of rice leaf sheaths associated with early-stage mortality of whitebacked planthopper eggs. This involves a response whereby air spaces around the eggs become filled with benzyl benzoate (Seino et al. 1996; Suzuki et al. 1996). The benzyl benzoate causes egg mortality either by directly killing the developing embryo or indirectly by affecting planthopper symbionts (symbiont-free eggs cannot complete embryonic development, Schwemmler, 1994 in Seino et al. 1996). Varieties with the ovicidal response, unlike those with other resistance mechanisms, appear highly durable, since they have been planted by farmers for millennia without any apparent virulence adaptation (Horgan, 2009). This may be due to their targeting of symbionts, which during the egg stage are restricted temporally and functionally from possibilities of adapting. However, there are several other possible explanations for the apparent durability of the ovicidal response, including the fact that japonica rice varieties are normally planted in regions where planthoppers cannot overwinter and where outbreaks are due to migratory populations (Bottrell and Schoenly, 2012).

5.0 Addressing the gaps in knowledge and improving research methodologies

Research into the role of symbionts in hopper virulence adaptation is still at an early stage. It has only been in the last 10 years that the nature of the bacterial endosymbiont community has been examined and that experimental studies have demonstrated a possible role for symbionts (YLS and bacteria) in mediating planthopper feeding on different rice varieties (Lu et al. 2004; Wang et al. 2010; Chen et al. 2011). The slow pace of discovery has been due to a poor understanding of each of the individual components in this complex and intricate three-way interaction. For example, there is a generally poor knowledge of the

mechanisms underlying rice resistance against planthoppers and leafhoppers: Whereas gene discovery has accelerated in recent decades, and some 79 resistance genes have been discovered, there is still a paucity of available information about what these genes actually do (Horgan, 2009; Fujita et al. 2013) and often by the time materials are available for research, virulent hopper populations have already developed (Myint et al. 2009b; Fujita et al. 2013). Furthermore, it has been difficult to determine whether identified symbionts (especially bacteria) have primary or secondary role in hopper survival. Further research is required. We make the following suggestions on how this research may be improved based on what is already known.

Better choice of host plants in selection studies: During the 1980s and early 1990s, at a time when few resistance genes had been identified, a series of studies examined the responses by planthoppers and leafhoppers to resistant donor varieties and compared resistant and susceptible varieties to determine probable candidate mechanisms (**Figure 1**). Much of that research used TN1 as a susceptible variety and Mudgo (*Bph1*) and ASD7 (*bph2*) as resistant varieties. Surprisingly, even though these varieties are known to be widely susceptible to planthoppers since the 1970s and 1980s, they have continued to be used in studies of virulence adaptation (Lu et al. 2004; Wang et al. 2010; Chen et al. 2011). Although switching of planthoppers between two susceptible varieties can give useful information (Chen et al. 2011), it would be more useful, at least for agriculture, to compare hopper population responses (including symbiont changes) over successive generations on highly resistant and highly susceptible varieties. To overcome problems with highly dissimilar rice genomes in different varieties, which leads to a complex of resistance mechanisms and feeding barriers, researchers can use near-isogenic rice lines (Fujita et al. 2010, 2013). Near-isogenic lines are lines developed using marker assisted selection that are generally similar to a recurrent susceptible parent (80-95% of genes are the same), but differ in possessing a gene

locus associated with resistance from the donor variety (Fujita et al. 2010). Responses by planthoppers and symbionts to selection on such lines can be better associated with specific resistance genes and their associated resistance mechanisms.

Replication of colonies in selection studies: During the 1980s, planthopper colonies selected on TN1, Mudgo and ASD7 at the International Rice Research Institute (IRRI) were central to screening and breeding for rice resistance. These colonies, designated as biotype 1, biotype 2 and biotype 3 were also later employed in studies of symbionts (Lu et al. 2004; Wang et al. 2010). Unfortunately, the biotype concept, which has been heavily criticized (i.e., Claridge and Den Hollander, 1982, 1983), cannot be adapted to field populations, and indeed relates only to the unreplicated laboratory populations at IRRI. Other selection studies, with different host plants have been conducted in general these also did not replicate selected colonies (i.e., Hirae et al. 2007; Peñalver Cruz et al. 2011; but see Alam and Cohen, 1998). Because colonies were never replicated, it has not been possible to determine whether observed trends in planthopper anatomy, amino acid composition, or symbiont community composition in past studies were a feature of the actual host plants on which the planthoppers had been reared, or were simply the result of directional selection and inbreeding. Future experiments should replicate selected colonies, preferably using hoppers collected from different locations as conducted by Alam and Cohen (1998).

Manipulation of symbiont communities: Although the selection of hoppers for several generations on a single variety constitutes a major investment to produce materials for virulence adaptation studies, reports of changes in the hoppers or their symbionts can remain too descriptive and ultimately suffer from the problems of cause-and-effect that are inherent to correlative studies (Wang et al. 2010). Some studies have used such materials for later manipulative experiments with symbionts: In particular, studies have compared symbiotic and aposymbiotic planthoppers for their responses to different rice lines (Lu et al. 2004; Chen

et al. 2011). Unfortunately, the obligate nature of endosymbiotic microorganisms has made it difficult to employ other types of manipulation. Aposymbiotic planthoppers are normally produced by heat-treatment (described above). This reduces YLS densities in the planthopper, but has unknown effects on the composition or abundance of bacterial symbionts. Indeed, because YLS include at least two species (Dong et. al 2011), heat treatment may selectively kill one or other species thereby affecting not only the abundance but also the composition of the YLS community. Care should be taken in seeking a mechanistic link between symbiont abundance and developmental abnormalities in planthopper eggs and nymphs since heat treatment may affect both the symbionts and the hoppers without any direct link between the two. For example, a recent report by Piyaphongkul et al. (2012) indicates that temperatures that were originally thought to affect YLS exclusively will also directly affect planthoppers: These authors suggest that the critical maximum temperature for brown planthopper nymphs is 34.9^{o}C. Although they did not include symbionts in their study, their claims do highlight the difficulty in distinguishing the effects of heat-shock from those of symbiont reduction. Clearly it is necessary to expand and improve the experimental methods available to researchers to allow the effective removal from planthoppers of 'native' symbionts and inoculation with 'novel' symbionts. Screening of antibiotics to remove symbionts and the development of techniques for micro-extraction and insertion of symbiont balls between planthopper eggs would be helpful for future research.

Further attention to bacterial symbionts: There are greater knowledge gaps associated with bacterial symbionts compared to YLS. In particular, it is still unknown how planthoppers and leafhoppers become inoculated with bacterial symbionts and whether these symbionts have primary or secondary roles in hopper survival. The occurrence of bacteria in the ovaries of planthoppers and leafhoppers (**Table 2**) suggests that they may be passed

through the egg – either on the egg surface or within the egg itself. However, bacteria have been associated with the planthopper salivary sheaths also (Wang et al. 2008a; Tang et al. 2010) suggesting that relations between the rice plant, the hoppers and bacterial symbionts may be more dynamic than for YLS. If virulence producing bacteria could be picked up from plants directly (perhaps after infestation by virulent planthoppers), then adaptation might be very rapid. Metagenomics can be employed to assess the complexity of the bacterial endosymbiont community in hoppers. The metagenomic approach considers the hopper as a community in which genomes belonging to other organisms, including bacteria and fungi, might be present (Vega and Dowd, 2005). For this purpose, meta-"omics" approaches such as metatranscriptomics, metaproteomics and metabolomics will be useful in profiling microbial activity (Xu, 2010). These technologies can also bypass the need for culturing symbiotic microorganisms as required when studying phylogeny and taxonomy. Because of the rapid pace of development of molecular tools available for research on bacteria, gaps in understanding the nature of bacterial symbionts will likely diminish rapidly; however, the use of novel tools should be linked with proper experimental materials, methods and manipulations as discussed above.

6.0 Concluding remarks

Throughout this review, we have suggested that interactions between the rice plant, planthoppers or leafhoppers, and their symbionts are complex. Our own preliminary results suggest that both symbionts and planthoppers will be involved in virulence and virulence adaptation and that their individual roles will likely depend on the nature of host plant resistance as well as the functions required for the hopper host vis-à-vis shifting from one susceptible variety to another or adapting to a widely available but highly resistant rice variety. Recent evidence indicates that these roles can change throughout selection and adaptation (Chen et al. 2011) and that symbionts (mainly YLS) might shift from being

beneficial during early stages of selection to become a drain on the insect host during later generations or where the insect is already largely adapted. Several process-related hypotheses remain to be tested: These include hypotheses aimed at two distinct levels of process – those that address the proximate mechanisms of adaptation, many of which are presented in **Figure 1**, and those that address the ultimate mechanisms of adaptation outlined at the beginning of the review (changes in symbiont communities, genetic or epigenetic shifts in symbionts or planthoppers). In terms of developing rice varieties and successfully deploying the varieties to reduce the rate of planthopper adaptation, both groups of hypotheses will be useful; however, there is a need to address those hypotheses that will help in the development of resistant varieties to better manage symbiont-mediated virulence adaptation. This will involve avoidance of host resistance that relies only on antibiosis (directed against nymphs) due to phloem nutrient deficiencies and by targeting not only the planthoppers and/or leafhoppers, but their endosymbiotic microorganisms as well. In general our knowledge of the role of symbionts in the dynamics between planthoppers or leafhoppers and their rice hosts is poor, in spite of the large and sustained investment that has been made in developing and deploying rice varieties with resistance to these insects. Nevertheless, in recent years, it has become clear that endosymbionts are essential in hopper nutrition and therefore, are expected to contribute greatly to virulence adaptation. Future research in this area has the potential to significantly change our approach to developing and deploying resistant rice varieties.

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CHAPTER 3

Varied responses by yeast-like symbionts during virulence adaptation in a monophagous phloem-feeding insect

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Abstract

This study examines the three-way interaction between symbionts, insect herbivores and their host plants during adaptation to resistant crop varieties. We conducted a long-term selection study (20 generations of continuous rearing) with a monophagous phloem-feeder, the brown planthopper (*Nilaparvata lugens* [Stål]), on several resistant rice (*Oryza sativa* L.) varieties. Planthopper fitness and the abundance of yeast-like symbionts (YLS) were monitored throughout the selection process. *N. lugens* populations collected from six regions in the Philippines adapted to the resistant varieties as noted by increasing body size, and increased egg-laying. Adaptation was partially through physiological and behavioral changes apparent during feeding: Planthoppers on resistant plants had relatively high levels of xylem-feeding compared with planthoppers on susceptible plants. YLS densities were highly dependent on the host rice variety. However, there were no consistent trends in YLS density: compared to densities in planthoppers on the standard susceptible variety Taichung Native 1 (TN1), YLS densities were consistently higher on PTB33 (resistant), similar on IR62 (resistant) but lower on IR65482 (moderately resistant) and IR22 (susceptible). Furthermore, YLS densities often remained the same despite improved planthopper fitness over generations. Our results do not support the hypothesis that changes in YLS density mediate planthopper adaptation to resistant varieties. However, slight reductions in YLS densities toward the end of selection on TN1, IR22 and IR62 may indicate that YLS have lower functional significance where varieties and environmental conditions are constant between generations.

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1.0 Introduction

The relative susceptibility or resistance of agricultural crops to herbivores forms a basis for the integrated management of several economically important herbivorous insects (Painter, 1951; Panda and Khush, 1995). Host plant resistance has been one of the principal focuses of public research into crop protection for the last 60 years (Painter, 1951; Panda and Khush, 1995; Porter et al. 2000; Smith, 2005; Stoner, 1996; Fujita et al. 2013). A recent emphasis on molecular biology and improved molecular tools has further increased attention toward host plant resistance (Fujita et al. 2013). Currently, several large-scale and long-term breeding programs have major components dedicated to the search for and application of resistance genes against major crop pests: i.e., Asian rice gall midge, *Orseolia oryzae* (Wood-Mason) (Lu et al. 2013); Russian wheat aphid, *Diuraphis noxia* (Kurdjumov) (Tolmay et al. 2013); Hessian fly, *Mayetiola destructor* (Say)(Garcés-Carrera et al. 2014); as well as rice leafhoppers and planthoppers (Fujita el al. 2013). However, it has become increasingly apparent that the ability of herbivores to rapidly adapt to resistant varieties (or resistance genes) represents a major constraint to the successful deployment and long-term utility of resistant varieties (Chen et al. 2011; Fujita et al. 2013; Ten Broeke et al. 2013; Wenger and Michel, 2013).

The development of rice varieties with resistance against the brown planthopper, *Nilaparvata lugens* (Stål), and the rapid adaptation by this insect to deployed rice varieties is among the best examples of both the successes and constraints in managing herbivores through host plant resistance. With the advent of the Green Revolution in the 1970s, *N. lugens* became a major pest of rice throughout Asia. Researchers at the International Rice Research Institute (IRRI) in the Philippines successfully developed varieties with resistance to the planthopper. In 1973, rice variety IR26 (containing the *Bph1* resistance gene) was released by IRRI and planted over a wide geographical area, particularly in Indonesia.

However, despite originally high levels of resistance, adapted *N. lugens* populations were already widespread by the late 1970s. IRRI together with national rice research institutes throughout Asia responded by releasing new varieties with new resistance genes (*bph2* and *Bph3*); however, *N. lugens* also quickly adapted to many of these varieties (Horgan, 2012; Fujita et al. 2013). With an increasing incidence of outbreaks in Asia since about 2002, researchers have intensified the search for resistance genes against *N. lugens*. A recent review has indicated that about 36 gene loci and several QTLs for resistance to *N. lugens* have now been identified (Fujita et al. 2013). However, screening studies conducted throughout South and South-East Asia indicates that already only a few of these genes are currently effective in reducing *N. lugens* fitness (Horgan et al. 2015). Furthermore, several laboratory studies have indicated that *N. lugens* and related insects (i.e., whitebacked planthopper, *Sogatella furcifera* [Horváth] and green leafhopper, *Nephotettix virescens* [Distant]) can adapt to resistant varieties within a few generations (5-12 generations: Pathak and Heinrichs, 1982; Claridge and den Hollander, 1982; Alam and Cohen, 1998; Hirae et al. 2007; Vu et al. 2014). The rapid pace of adaptation suggests that mechanisms other than genetic selection are likely to play a major role in virulence and adaptation by *N. lugens* and other phloem-feeding insects. One adaptation mechanism that has gained increasing attention is symbiont-mediated facilitation of feeding or digestion (Ferrater et al. 2013; Wenger and Michel, 2013; Hansen and Moran, 2014). Endosymbionts have been considered to facilitate feeding and determine host plant preferences in herbivores (Chung et al. 2013, Ferrater et al. 2013; Wenger and Michel, 2013; Hansen and Moran, 2014). Therefore, endosymbionts could have the potential to accelerate adaptation to resistant rice varieties by promoting survival on poor quality food plants such as resistant varieties at the early generations of selection. This research has received little attention as indicated in these few available studies (Lu et al. 2004; Tang et al. 2010; Chen et al. 2011).

Several endosymbionts have been identified from *N. lugens*. These include diverse mutualistic eukaryotic and prokaryotic organisms that live inside the insect body and are associated with the host's feeding, development and reproduction (Ferrater et al. 2013). Of these, the yeasts and yeast-like symbionts (YLS) have received most research attention. YLS are found in the abdominal fat bodies, ovaries and eggs of *N. lugens* (Ferrater et al. 2013). YLS (including genebank accession no. AF267233) that occur in the fat bodies of *N. lugens, S. furcifera* and *Laodelphax striatellus* (Fallén) are contained in specialized mycetocytes and are inherited through the egg (Ferrater et al. 2013). Recently, Dong et al. (2011) identified at least two species of YLS in the fat bodies of *N. lugens* and called these *Cryptococcus* (*Cryp*)-like and *Pichia*-like symbionts. However, other species may also occur (Zhang et al. 2009; Pang et al. 2012). Clear evidence exists that links YLS to the facilitation of feeding in the rice planthoppers *N. lugens* and *Laodelphax striatellus* (Fallén): Nutrient-deficient phloem sap is supplemented with essential amino acids by YLS during recycling of metabolic wastes (Noda et al. 1979; Wetzel et al. 1992; Sasaki et al. 1996; Hongoh and Ishikawa, 1997; Wilkinson and Ishikawa, 2001; Noda and Koizumi, 2003).

At least three studies have indicated that endosymbionts (YLS: Lu et al. 2004; Chen et al. 2011; bacteria: Tang et al. 2010) respond to switching between feeding plants (rice varieties) by their insect hosts. Lu et al. (2004) suggested that virulence of *N. lugens* populations to resistant rice varieties was strongly related to the abundance of YLS. In that study, there were significant decreases in YLS abundance initially upon transferring *N. lugens* from susceptible to resistant rice varieties and a gradual increase in YLS abundance as the planthoppers approached full adaptation. Chen et al. (2011) observed that YLS improved nymph survival during some (early) generations of selection on resistant varieties, but that in later generations the YLS represented a 'drain' on planthopper fitness. Furthermore, Tang et al. (2010) indicated that endosymbiotic bacterial communities may shift in response to

selection on different rice varieties. Unfortunately, these studies give limited information as to the potential diversity of responses by the symbionts of *N. lugens* to shifts in host feeding on resistant varieties, particularly since all three studies used the same rice varieties (Taichung Native 1 [TN1], Mudgo and ASD7) despite the fact that each of these varieties was already susceptible to *N. lugens* at the time the research was undertaken as a result of widespread planthopper adaptation.

To expand knowledge on the three-way interaction between symbionts, *N. lugens* and rice plants, we conducted a long-term selection study of *N. lugens* on several resistant varieties. Previous studies suggest that symbiont numbers would be lower for *N. lugens* on resistant varieties than on susceptible varieties and that once adapted to a resistant variety, symbiont abundance would be similar in adapted hoppers on resistant varieties and non-adapted hoppers on susceptible varieties (Lu et al. 2004; Chen et al. 2011). However, we expanded the range of varieties (including currently resistant varieties and also changing *N. lugens* between two susceptible varieties) to examine whether patterns observed in previous studies have been general or were specific to the varieties in question. We also carefully followed patterns in YLS abundance throughout the selection process to gain a better understanding of the potential changing roles of YLS as *N. lugens* better adapts to its feeding plant. To our knowledge this is the largest comparative study of *N. lugens* and YLS responses and adaptation to resistant host plants.

2.0 Materials and methods

2.1 Insect Populations

Brown planthopper, *Nilaparvata lugens* (Stål), populations were collected from Philippine rice paddies between September and November of 2009. The insects collected consisted of about 200-500 (50 at Iloilo) individuals from each of six geographic locations (North Luzon [17.00° N, 122.00° E], Laguna [14.17° N, 121.33° E], Bicol [13.50° N, 123.33°

E], Mindoro [13.00° N, 121.08° E], Iloilo [10.72° N, 122.57° E], and Mindanao [6.17° N, 125.00° E]). The collection sites represent areas that are widely cultivated for rice production in the Philippines (Launio et al. 2008). The planthopper founding populations were maintained on 45-day old plants of the highly susceptible rice variety Taichung Native 1 (TN1) in aluminum wire mesh cages of 91.5 cm × 56.5 cm × 56.5 cm (H×L×W) (**Figure 1A**) in a greenhouse at the International Rice Research Institute (IRRI), Los Baños, Philippines. Temperatures in the greenhouse ranged between 26 and 45^{o}C over the course of the study, with natural daylight regimes (light from ca. 6am to 6pm throughout the year). The colonies were continuously fed with TN1 for 3 to 5 generations. After five generations, ca. 500 gravid females were taken from the main cage of each of the six populations and ca. 100 each placed on five differential rice varieties (**Table 1**) to initiate colonies for selection and monitoring (a total of 30 colonies). A differential variety is a variety for which insect populations demonstrate (or have demonstrated) marked differences in preference or performance, often indicating the presence of major resistance genes.

2.2 Plant materials

Prior to initiating the selection study, a range of differential varieties were examined for their resistance to *N. lugens*. From an original group of 16 varieties, three varieties were found to be moderately (IR65482-4-136-2-2 [henceforth IR65482]) or strongly (PTB33 and IR62) resistant. Pre-screening also revealed that all six *N. lugens* populations were adapted to varieties with the *Bph1* and *bph2* genes for resistance (Horgan and Bernal, unpublished data). Details of the differential varieties are presented in **Table 1**. The breeding line IR65482 contains the resistance gene *Bph10* derived from the wild rice *Oryza australiensis* Domin (Ishii et al. 1994; Fujita et al. 2013).

Figure 1. Types of cages used in rearing *Nilaparvata lugens.* A) aluminum wire mesh cage with 45-day old rice on soil medium B) plexiglass cage with rice seedlings on soilless, nutrient medium.

Table 1. Rice differentials used in the selection experiments with *Nilaparvata lugens.*

Rice variety	Origin	Resistance status	Resistance sources	Source references
Taichung Native 1	Variety released in 1960 in Taiwan	Susceptible	None	De Datta (1981)
IR22	Variety released in 1969 in the Philippines	Susceptible	None	Khush and Virk (2005); Brar et al. (2009)
IR65482-4-136-2-2	Donor line from IRRI Philippines	Moderately resistant	*Bph10* from *Oryza australiensis* Domin	Ishii et al. (1994)
IR62	Variety, released in 1984 in the Philippines	Resistant	*Bph3* from PTB33	Brar et al. (2009)
PTB33	Traditional Indian landrace	Resistant	*bph2*, *Bph3* and QTLs	Sidhu and Khush (1978); Jairin et al. (2007); Yadavalli et al. (2012)

PTB33 is a traditional Indian variety considered among the most resistant rice varieties to Philippines populations of *N. lugens.* It has been used extensively in breeding programs aimed at increasing rice resistance to biotic stresses (Khush and Virk, 2005). The genetics of resistance in PTB33 is still not fully understood, but is likely governed by a number of major resistance genes (including *bph2* and *Bph3*) (Sidhu and Khush, 1978) and several minor genes (Yadavalli et al. 2012). IR62 is a modern rice variety released by IRRI in 1984. The variety is highly resistant to *N. lugens* from Laguna. Its resistance has been attributed to the *Bph3* gene that was likely obtained from PTB33 (Khush and Virk, 2005; Peñalver Cruz et al. 2011). IR65482 and PTB33 are not commercially grown by rice farmers. IR62 is not commonly grown in the Philippines (<1% adoption among farmers - Peñalver Cruz et al. 2011), although it is popular among some farmers in Cambodia (P. Virk, personal communication). We used a further variety, IR22, as a susceptible control during the experiments. IR22 is thought to contain no major resistance genes (Brar et al. 2009).

All seeds of each of the five varieties were acquired from IRRI: TN1 and PTB33 seeds were acquired through the International Network for Genetic Evaluation of Rice (INGER). The IR varieties (IR22, IR62 and IR65483) were acquired through the Plant Breeding, Genetics and Biotechnology Division. The seeds were planted and bulked-up in a screenhouse facility at IRRI to attain sufficient supply for the study.

2.3 Maintenance and monitoring of *N. lugens* populations on differential varieties

The 30 experimental colonies were continuously maintained in transparent plexiglass cages (30cm × 25cm × 30 cm; H×L×W) during 20 generations of selection. The cages were maintained in an insectary with a 12h:12h L:D regime and a constant temperature of ca. 26^{o}C. For screening, the seeds were surface sterilized with 1% (v/v) sodium hypochlorite for 10 minutes, soaked for 24 hours in cups with filtered water and placed in a chamber covered with black cloth. After soaking, the pre-germinated seeds were washed with filtered water and sown on shallow plexiglass trays lined with two layers of moistened cotton gauze and soaked with excess Yoshida soilless nutrient solution (Yoshida et al. 1976). The trays could be inserted neatly into the plexiglass cages. Seedlings of ≥ 7 days old were used as feeding plants during the study. The exposed seedlings were changed every week with fresh seedlings. For the purposes of this study, and to improve the robustness of the experiments, we regard each group of colonies (e.g., Laguna on TN1, Laguna on IR22, Laguna on PTB33, etc.) as experimental 'colony-origin' blocks and the individual colonies as replicated experimental units (see Ferrater et al. 2013). Colonies were maintained on plexiglass cages (**Figure 1B**) in the insectary with host-plants randomized within colony-origin blocks. The hopper life cycle in the colonies was divided into two phases: egg-laying by adults and egg-adult survival. For each generation, initially 100 gravid females were maintained on the seedlings for 5 days for egg-laying, after which they were removed and the eggs allowed to develop. Females that were not caged for egg-laying were air dried for about 2 hours in the

laboratory to remove excess surface water and later dissected to estimate the abundance of YLS (see below). Once the first 200-300 nymphs emerged, the remaining, unhatched eggs were removed from the cages by changing the feeding plants. Colonies were observed until fifth instar nymphs were about to molt to adults (approximately 15-20 days), at which time, 100 individuals were collected and transferred to new cages to begin a subsequent generation. This was repeated during 20 generations (approximately 24 months). Planthoppers from each colony, at each generation, were monitored for their ability to survive and develop on the differential varieties using the following parameters and bioassays:

Adult biomass: To determine the performance of the planthoppers on each variety, six newly molted adult females were randomly collected from the cages at each generation. The insects were air-dried (to remove excess surface water) for two hours and weighed. This was recorded as the adult fresh body weight.

Honeydew production: The amount and nature of honeydew excreted by gravid female *N. lugens* was monitored using the method of Pathak and Heinrichs (1982). Two newly-emerged adults were starved for 1 hour before being placed together to feed on 20-day old plants for 24h in specially prepared plastic chambers that restricted the hoppers to within 5cm of the base of the plants. The chambers were placed on top of filter paper, neatly fitted around the plant shoot (**Figure 2A**). The filter papers had been treated with bromocresol green. Bromocresol green indicates the nature of the honeydew as coming from the phloem (basic reaction indicated by blue-rimmed spots) or xylem (acidic reaction indicated by white spots)(Yesuraja and Mariappan, 1991). The area of excreted honeydew spots on the bromocresol-treated filter paper was measured using Image J software version 1.48 (National Institutes of Health, USA).

*Oviposition performance.*The number of eggs laid on each variety was determined by confining pairs of adult *N. lugens* (male and female) on 20-day old plants for 8 days. Plants

were produced from seed in size zero clay pots (7 cm × 11 cm; H × D) each enclosed in a cylindrical mylar cage (61cm × 10.5cm; H ×D)(**Figure 2B**) with a mesh side window and top for ventilation. After 8 days, the insects were removed and the plants were collected and frozen at -20^{o}C. These plants were later dissected and the number of eggs deposited on each plant was counted under a stereomicroscope (10× magnification).

Oviposition bioassays were carried out in a greenhouse at temperatures ranging from 25-45^{o}C, whereas honeydew production was measured in a controlled temperature room at 26ºC. All bioassays were repeated twice for each of the 30 colonies to increase precision of monitoring during each generation. Bioassay units were arranged as a completely randomized design.

2.4 Changes in YLS density during virulence adaptation

Each colony was examined for changes in YLS abundance per unit insect weight (henceforth YLS density) over the course of selection. YLS densities were estimated from adult females collected prior to egg-caging at generations 1, 3, 5, 8, 10, 13, 15, 18 and 20 following the method of Chen et al. (2011). Six adult females were weighed (wet weight) and homogenized in 500µl physiological saline solution (0.9% NaCl). An aliquot of 10µl was transferred to a haemocytometer cell counter and suspended materials identified as YLS (**Figure 3**) were counted under a compound microscope (40× magnification). YLS abundance was divided by the weight of the females to indicate density.

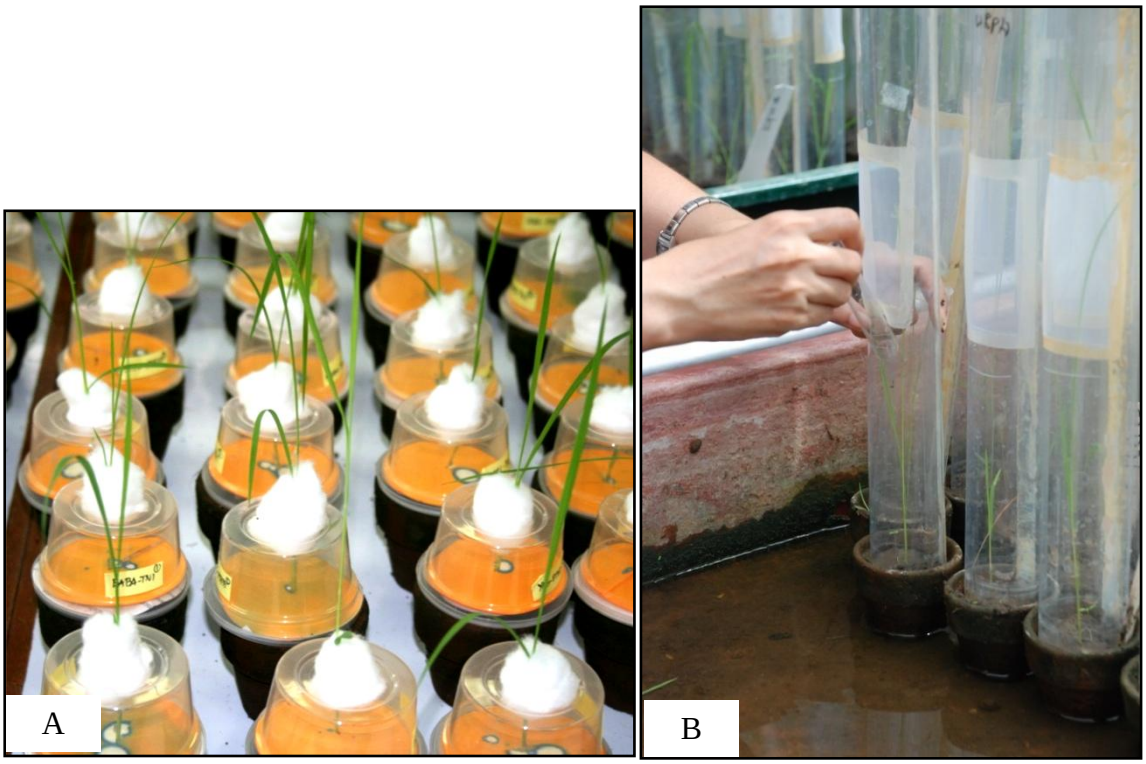

Figure 2. Perfomance bioassay for *Nilaparvata lugens* A) honeydew set-up B) oviposition set-up, each plant is enclosed in a cylindrical mylar cage.

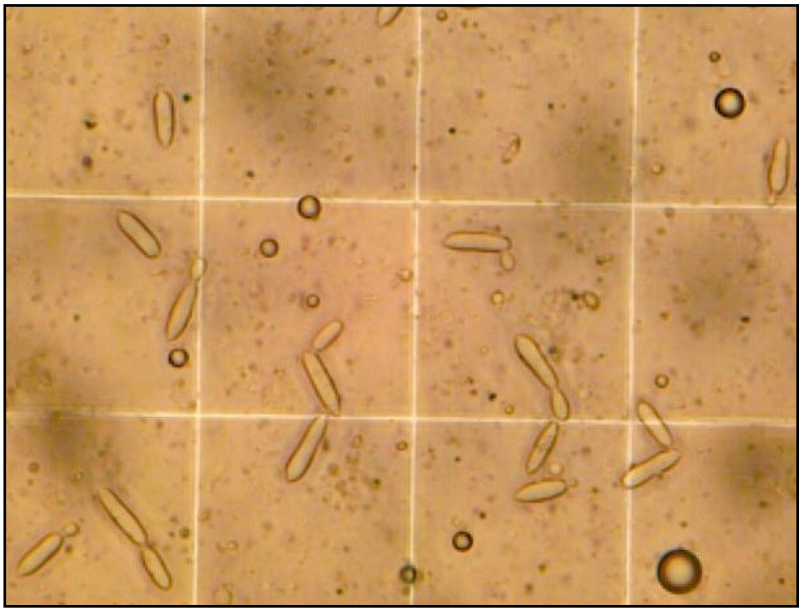

Figure 3. Photomicrograph of the yeast-like symbionts (YLS) at 400× magnification.

2.5 Data analysis

Fitness parameters estimated for planthoppers on IR22, IR65482, IR62 and PTB33 were standardized for each generation and block (origins) against corresponding planthoppers reared on TN1. This was carried out to standardize for fluctuations in environmental conditions over the course of selection and during the conducting of fitness bioassays. Changes in fitness parameters over the selection period were examined using repeated general linear models (GLM). Tukey tests were used for post hoc analyses. Model parameters included generation of selection, host-plant variety and interactions. Only significant interaction terms are presented in the results. Colony origin was originally incorporated as a block factor, but had no effect during any of the analyses and was finally removed. Honeydew production was expressed by xylem-derived honeydew as a proportion of total honeydew production. Xylem is considered a less favourable source of nutrition for planthoppers compared to phloem, such that xylem-feeding is thought to indicate antibiotic resistance of the host plant. By standardizing to total honeydew production, we controlled for variability in the size of adults throughout selection and for the effects of environmental conditions on planthopper feeding rates. Trends in the absolute densities of YLS over the selection period were examined for the best model fit using Sigma Plot 11.

3.0 Results

3.1 Responses by *N. lugens* to selection on differential varieties

Planthoppers reared on resistant varieties generally increased xylem feeding compared to colonies reared on TN1. Production of xylem spots increased during the early generations after the colonies were moved from their natal variety (TN1) to the four new varieties (**Figure 4**, **Table 2**). Xylem feeding generally remained relatively high on the resistant varieties throughout selection (**Figure 4B,C,D,Table 2**) but was similar for colonies feeding on TN1 and IR22.

Egg-laying generally increased over the course of selection with females after about 15 generations on the resistant varieties approaching, but never exceeding, the number of eggs laid by TN1-reared planthoppers (**Figure 4F,G,H, Table 2**). Planthoppers reared on IR22 laid the largest numbers of eggs, and this was significantly higher than planthoppers reared on PTB33 and IR62 (**Figure 4E-H, Table 2**).

Planthopper body size (measured as adult fresh weight) generally increased over the course of selection, in many cases attaining similar sizes to adults from the TN1 colonies (**Figure 4I-L, Table 2**). Planthoppers reared on PTB33 were generally the smallest, whereas planthoppers on IR22 and IR65482 often attained higher weights than those from TN1 (**Figure 4I-L, Table 2**). Variation in planthopper weight over the course of selection, particularly on PTB33 resulted in a significant (generation*host plant) interaction (**Figure 4I-L**, **Table 2**).

3.2 Density responses by YLS to host selection on differential varieties

The densities of YLS changed significantly over the course of selection (**Figure 4M-P**, **Table 2**). However, density trends over the course of selection varied considerably between planthoppers reared on the different host plant varieties. In particular, YLS densities in PTB33-reared planthoppers increased toward the end of selection, exceeding numbers estimated for TN1, whereas on IR62, symbiont density was generally similar to densities estimated for planthoppers on TN1. Overall, the magnitude of deviations in YLS density from the TN1 controls was greatest for planthoppers on PTB33 and lowest for IR22; planthoppers on IR62 and IR65482 were intermediate in YLS density and more similar to TN1 (**Figure 4M-P, Table 2**).

The absolute density of YLS in planthoppers reared on each of the rice varieties tended to decline toward the end of the selection experiment. This occurred even among the TN1-reared colonies. Trends best approximated a quadratic curve for TN1 ($F_{2,39} = 9.508$, $P <$

0.001, R^2 = 0.34 – **Figure 5A**), IR62 ($F_{2,44}$ = 16.185, P , 0.001, R^2 = 0.44, **Figure 5D**) and IR22 ($F_{2,43}$ = 6.318, P = 0.004, R^2 = 0.24 – Figure 2B). There were no apparent trends in symbiont density for planthoppers reared on IR65482 or PTB33 (**Figure 5C,E**).

Table 2. Results from repeated measures GLM of the effects of exposed variety, generation and their interaction on *N. lugens* feeding and fitness parameters, and on the density of yeast-like symbionts (see **Figure 4**).

Sources of variation	Xylem/total honeydew			Eggs per g/plant			Adult weight			YLS density		
	Df	F	P[1]	df	F	P[1]	df	F	P[1]	df	F	P[1]
Between subject effects												
Variety	3	26.496	**<0.001**	3	6.720	**<0.001**	3	82.999	**<0.001**	3	161.305	**<0.001**
Df	20			20			20			20		
Within subject effects												
Generation	19	3.511	**<0.001**	17	5.243	**<0.001**	17	8.199	**<0.001**	7	5.321	**<0.001**
Generation × variety	57	0.723	0.933	51	0.735	0.910	51	1.967	**<0.001**	21	1.123	0.331
Df	380			340			340			140		

1: P-values in bold font indicate significant differences; homogenous groups for between subject variety effects are indicated in **Figure 3**.

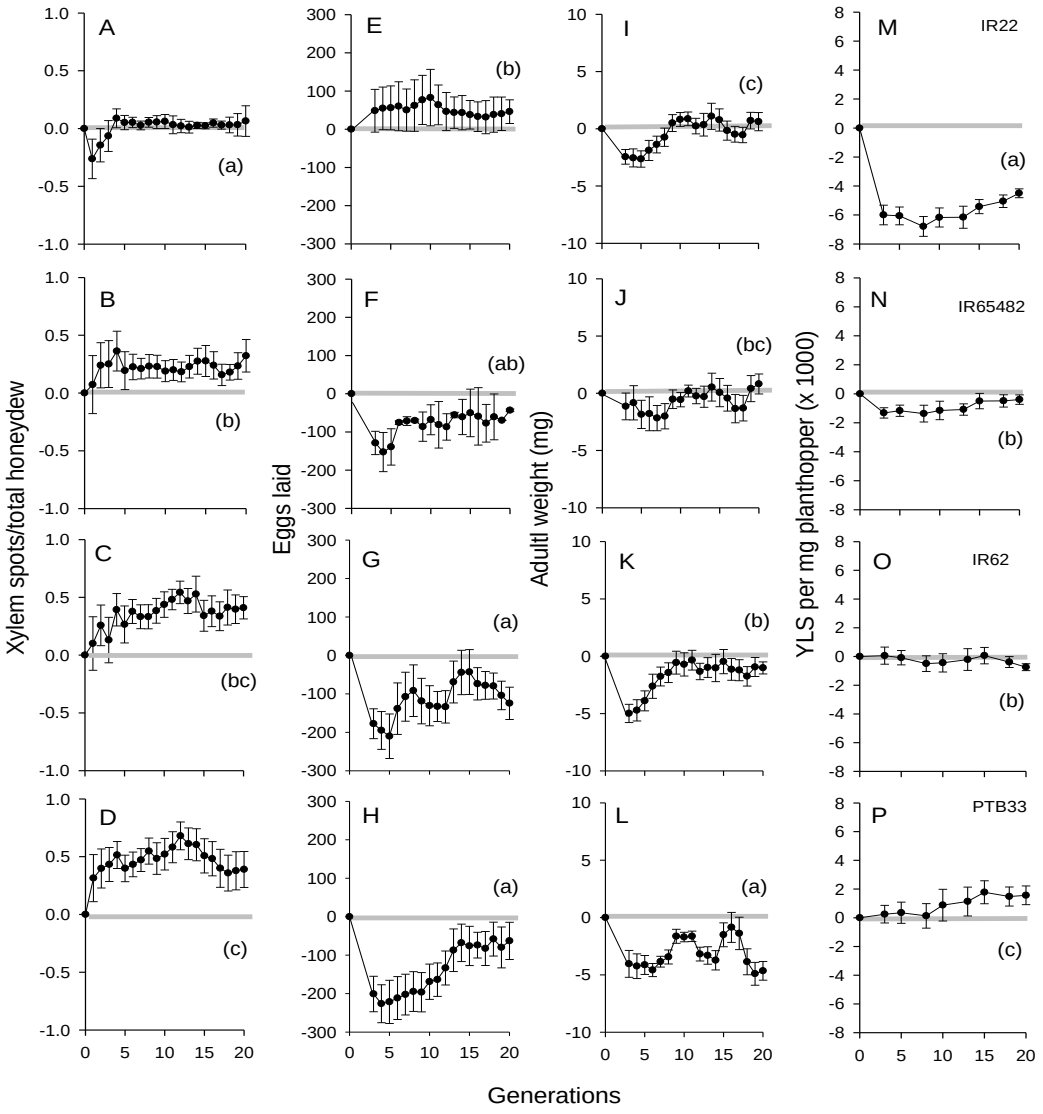

Figure 4. Indicators of average *Nilaparvata lugens* colony fitness during selection on IR22 (A,E,I,M), IR65482 (B,F,J,N), IR62 (C,G,K,O), and PTB33 (D,H,L,P). Colony fitness was monitored throughout selection and compared against the susceptible standard TN1 (grey lines indicate no difference and minus values indicate that the measured parameters, as on y-axes, were less on the exposed plants than for planthoppers on TN1). The fitness parameters recorded (all standardized to corresponding measures on TN1) were honeydew composition (proportion of xylem-derived honeydew/total honeydew production)(A-D), number of eggs laid per female (E-H), adult fresh weight (I-L), and density of yeast-like symbionts (M-P). Means are from 6 colonies (n = 6) and are presented as 3-point moving averages. Bars indicate standard errors. Letters in parentheses indicate homogenous groups of varieties.

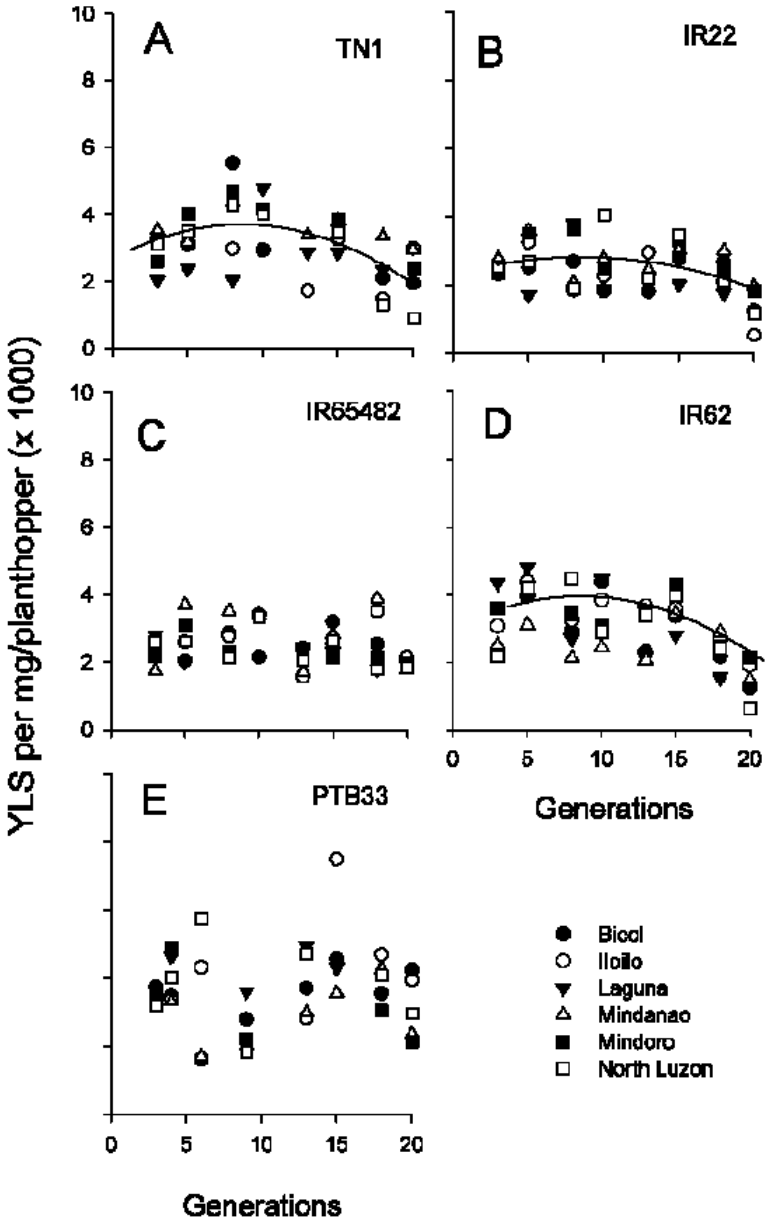

Figure 5. Changes in the densities of yeast-like symbionts in *Nilaparvata lugens* during selection on rice lines. The densities of yeast-like symbionts in planthoppers reared on the standard susceptible variety TN1 are indicated (A) together with abundances on the four test differentials IR22 (B), IR65482(C), IR62 (D) and PTB33(E). Symbols indicate colony origin as Bicol (solid circle), Iloilo (open circle), Laguna (solid triangle), Mindanao (open triangle), Mindoro (solid square) and North Luzon (open square). Curves indicate the best fit to data: A $y = 2.15 + 0.324x - 0.0168x^2$; B $y = 2.16 + 0.3143x - 0.008x^2$ and D $y = 2.86 + 0.205x - 0.0131x^2$.

4.0 Discussion

Adaptation by planthoppers to resistant rice varieties has been well documented from several laboratory studies as well as from field monitoring. Planthoppers, continuously reared on resistant varieties have been shown to gradually increase in fitness over successive generations until they reach similar fitness levels to hoppers reared on susceptible varieties. Planthoppers generally improve feeding efficiency, nymph survival and weight gain, as well as egg-laying through generations of selection following host switching (Pathak and Heinrichs, 1982; Claridge and den Hollander, 1982; Alam and Cohen, 1998; Peñalver Cruz et al. 2011). Our results are generally consistent with previous studies that indicate gradual improvements in planthopper fitness through selection on resistant varieties. However, we noted some behavioural changes in the planthoppers that suggest that adaptation did not result in planthoppers feeding equally on susceptible and resistant hosts; for example, on resistant varieties the planthoppers continued to probe and feed on xylem to a greater extent than observed among planthoppers on susceptible varieties. Furthermore, our study indicated that changes in YLS density over time were different for planthoppers feeding on different varieties and did not match the trends noted in previous studies with the varieties Mudgo and ASD7 (Lu et al. 2004; Chen et al. 2011).

4.1 Adaptation by *N. lugens* to resistant rice varieties

Our results are typical of those from selection studies in that the planthoppers' ability to feed on their host varieties gradually improved during selection; however, we noted that the nature of planthopper feeding changed as these adapted to resistant varieties: Planthoppers on resistant varieties gradually increased production of acidic honeydew during the first five generations (indicating that they were feeding on xylem, which is largely considered as nutrient deficient [Andersen et al. 1989]) and continued to feed on xylem throughout the 20 generations of monitoring. Xylem feeding is normally considered an

indicator of host plant resistance for *N. lugens*, which is a phloem feeder (Yoshihara et al. 1980; He et al. 2010). The predominance of xylem-derived honeydew suggests that the adaptation by the hoppers to feed on resistant varieties in the present study included a physiological or behavioural shift toward xylem acceptance or xylem feeding. Xylem sap consumption was first observed in dehydrated alate aphids (Spiller et al. 1990), and thus was associated with replenishing water following the dehydration period prior to flight (Powell and Hardie, 2002). However, a recent study has shown that both well hydrated alate and apterate aphids ingest xylem sap under certain conditions (Pompon et al. 2010), and that there are other reasons, in addition to dehydration, for mixing phloem and xylem sap. The consumption of xylem sap is considered a general response by insects to osmotic stresses. The low osmolarity of the xylem allows phloem-sap feeders to regulate their osmotic potential when the osmotic potential of the phloem increases due to sugar or non-sugar contents (Pompon et al. 2011). In *N. lugens* feeding on rice, a balance between nutritional and defensive compounds appears to influence feeding preference and nymphal performance (Chen, 2009). It is possible that a xylem-phloem mix during feeding by *N. lugens* may be a strategy to dilute feeding deterrents in the phloem to overcome rice resistance.

We used adult weight to indicate feeding and developmental success on the different host plants. As expected, adults generally increased gradually in average weight with time since transfer to the new variety. It is noteworthy that this gradual increase in adult weight was apparent for planthoppers reared on the susceptible variety IR22 as well as on the resistant variety IR62 and demonstrates the barrier to variety switching in *N. lugens*, even when switching between two apparently susceptible varieties (in this case TN1 and IR22). Adult weights were generally similar to those on TN1 for hoppers reared on IR65482, but fluctuated over selection on PTB33. The size of planthopper populations tended to remain low on PTB33 throughout selection (data not presented), despite the occurrence of large

individuals during some generations. This pattern of adaptation and regression may be due to the strong resistance of PTB33 (associated with several genes and QTLs: **Table 1**). It also suggests that well adapted individuals often failed to provide successful progeny to successive generations possibly due to experimenter errors during colony manipulation or because of unforeseen fitness costs. Whatever the reasons, this pattern of adaptation likely reflects the nature of adaptation in field populations where other selection forces and possible trade-offs influence the success of adapting populations to provide progeny for future generations. It also runs counter to the concept of stable biotypes in wild populations. We noted that *N. lugens* feeding on PTB33 tended to feed higher (on leaves) on the host plant than is normal (on the leaf sheath) and that the planthoppers fed on xylem about 50% of the time. Females generally laid more eggs on IR22 than on TN1 throughout selection, and gradually increased on the resistant varieties to approach egg numbers typical for planthoppers on variety TN1.

4.2 YLS and adaptation

In the study by Lu et al. (2004), *N. lugens* were reared on three host varieties (TN1, Mudgo and ASD7) for four generations. The authors also monitored YLS abundance (not density as in the present study) and found that abundance declined successively during the first two generations on the resistant varieties (Mudgo and ASD7) but then increased to reach similar levels as on TN1 by the fourth generation. The similarity of responses on two resistant varieties with different resistance genes (Mudgo – *Bph1* and ASD7 – *bph2*) and using distinct planthopper populations (designated biotype 2 and biotype 3) suggested that the YLS played a role in adaptation. Furthermore, reduction of YLS abundance through heat treatment (aposymbiotic) had more severe consequences (in terms of nymph duration and adult fecundity) for hoppers on resistant varieties than on TN1 (Lu et al. 2004). Similarly, reduction of YLS abundance through heat treatment in a study by Chen et al. (2011) reduced

nymph weight on TN1, Mudgo and ASD7 for *N. lugens* from colonies reared successively for 8 generations on the same hosts. However, in the same study, the authors noted that by the 11th generation the symbionts appeared to represent a drain for the planthoppers since aposymbiotic hoppers attained heavier weights than symbiotic hoppers from the same colonies and on the same host varieties. These studies suggest that symbiont abundance is initially low following host switching from TN1 to either Mudgo or ASD7, gradually builds up during selection to reach similar levels as on susceptible varieties, and at some stage after several generations may become a drain to the insect host. Our results with different host plant varieties indicate that such a pattern through the course of selection is not general. We found YLS densities to decline quickly on IR22 relative to TN1. Densities were also generally lower on IR65482 relative to TN1, but gradually increased over the course of selection, and in the highly resistant variety PTB33 they were generally higher than for TN1. Furthermore, on the highly resistant variety IR62, YLS densities were similar to TN1 throughout selection. Furthermore, these trends were largely consistent for replicated colonies derived from six founder populations collected throughout the Philippines.

The decline in YLS densities recorded for TN1, IR62 and IR22 towards the end of the selection experiment might indicate that the symbionts were not as necessary for planthopper survival at that time compared with the beginning of the experiment. These results therefore support the symbiont-drain hypothesis (Chen et al. 2011). Chen et al. (2011) noted that heat-treated planthoppers that had been continually reared on a single host variety during 11 generations performed better than control hoppers (non-heat treated) on the same varieties. Therefore, taken together, these results demonstrate that YLS densities respond to planthopper feeding on different rice varieties in markedly different ways and that YLS density may play a role in allowing hoppers to switch from one variety to another providing nutrients where successive generations are likely to encounter a diversity of host plant

varieties, but may be less important where varieties are constant. Furthermore, the relatively low YLS densities observed among colonies reared on IR22, suggest that the variety, although a very favourable host for *N. lugens*, may be less suitable for YLS. However, we examined only one aspect of the YLS community – density. Responses by the YLS community (which may consist of two or more YLS species) might also be manifested in shifts in the relative abundance of individual yeast species. Further detailed studies of the species composition of the YLS communities as they change over generations of planthopper feeding would clarify whether particular symbiont species actually play a role during adaptation.

4.3 Resistance mechanisms and insect and YLS responses

Several resistance genes have been identified in rice against planthoppers and leafhoppers (currently about 80 genes); however, in general very little is known about the mechanisms underlying resistance in rice (Horgan, 2009; Fujita et al. 2013). Mechanisms of resistance related to the *Bph3* gene present in IR62 and PTB33 have been studied by Saxena and Okech (1985), and Stevenson et al. (1996). Saxena and Okech (1985) indicated that volatiles emitted from Rathu-Heenati (*Bph3* gene) decreased settling and feeding and increased planthopper mortality. However, as indicated by Ferrater et al. (2013), symbionts are unlikely to play a role in planthopper adaptation to volatile-based defenses. Stevenson et al. (1996) found that the phloem of Rathu-Heenati and derived rice lines had higher concentrations of C-glycosidic flavonoids than in susceptible varieties. In feeding trials they found that high concentrations of one of these flavonoids - schaftoside - caused mortality of planthoppers. Schaftoside is thought to act as an antifeedant. The same or similar antifeedants in IR62 and PTB33 may have determined the low weight gain of planthoppers in our selection experiment and would have affected YLS in different ways depending on whether the antifeedant functions as an antidigestive, antinutritive or antiabsorbative. The similar

densities of YLS in IR62-reared and TN1-reared hoppers and the high densities of YLS in PTB33-reared hoppers suggest that the hoppers could supply sufficient nutrients to the symbionts even when the hoppers themselves failed to gain weight and had low fitness. These results indicate a decoupling of effects where the YLS were likely unaffected by the host plant defense mechanisms, particularly in PTB33, but where the hoppers were directly targeted by the plant's defenses. This suggests that the YLS likely provided sufficient nutrients to compensate for the poor food quality of the rice variety and allowed the planthoppers to survive on the resistant host; however, the trends do not support the hypothesis that YLS mediated adaptation – since YLS abundance remained relatively stable throughout selection on IR65482 and PTB33, and was generally high for planthoppers on PTB33 despite relatively poor adaptation. The notable drop in YLS abundance on IR22 despite high fitness of planthoppers on this variety also indicates that there is no predictable relationship between YLS abundance and fitness.

5.0 Conclusions

Planthoppers have a large capacity to adapt to resistant rice varieties. Physiological and behavioural changes can support planthopper feeding on resistant varieties including increased xylem feeding or changes in feeding locations. Previous studies have suggested that YLS and bacterial symbionts can play a role in virulence adaptation in *N. lugens*. Furthermore, it has been proposed that the functions of YLS and the relationship between the YLS and the planthoppers could change over generations of selection. Our results indicate that there are no general trends in symbiont abundance or density over the course of adaptation and that planthoppers on susceptible varieties (i.e., IR22) can have low densities of YLS while hoppers on resistant varieties can have very high YLS densities. YLS densities cannot therefore be correlated with adaptation nor can the trends (changes over time or relative densities) be predicted. However, in our experiments, on some varieties YLS

densities tended to increase at the beginning of selection but decreased after several generations. This was consistent with a decreasing role for symbionts in constant environments and suggests that a key role of YLS may be to support shifts by planthoppers between different rice varieties. Future studies should determine the species composition of the YLS communities and whether all symbiont species respond equally to changing host plant quality.

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CHAPTER 4

Responses by yeast-like symbionts to host-plant switching in a monophagous phloem-feeding insect

Jedeliza B. Ferrater, Finbarr G. Horgan

Abstract

Insect herbivores form symbioses with a diversity of prokaryotic and eukaryotic microorganisms. For many herbivores, endosymbionts play a major role during host feeding on nutrient poor diets – including phloem. Furthermore, symbiont-herbivore associations have been implicated in feeding preferences by host races (mainly aphids) on different plant species. However, the role of symbionts in mediating herbivore preferences between different varieties of the same plant species has received little research attention, despite the implications for virulence adaptation to resistant crops. This study investigates the link between densities of yeast-like symbionts (YLS) and host-plant switching in populations of the brown planthopper, *Nilaparvata lugens* (Stål), that were selected on various rice lines, differing in resistance towards herbivores. Planthopper fitness (nymph weight) declined when YLS densities were depleted through heat treatment. Compared to normal symbiotic planthoppers, the depletion of symbionts did not generally change the relative fitness of planthoppers (each reared on a single natal host) when switched to feed on a range of different rice lines (exposed hosts). In most cases, this occurred in spite of differences in YLS density responses to the different hosts. These results suggest that changes in YLS density play only a minor role during host plant switching by the brown planthopper and that this role is independent of major anti-herbivore resistance genes.

Submitted for publication

1.0 Introduction

Individuals of the same plant species often occur together as patches within their natural habitats. The degree of isolation between patches determines the magnitude of reproductive barriers between individual plants in different patches (McNeilly and Antonovics, 1968; Loveless and Hamrick 1984; Husband and Barrett, 1996). Barriers to gene flow, spatial variability in the selective forces between different patches over time, and variability in the environmental conditions experienced at different patches can result in plants that are phenotypically distinct at different locations (Zangerl and Berenbaum, 2003; Berenbaum, 1988; Horgan, 2009). Phenotypic differences between plants have been noted to determine feeding preferences in oligophagous and monophagous insects and can act as a barrier to switching by insects between individual plants of the same species. Such barriers can result from differences in plant defenses among populations as has been shown for wild plants (Berenbaum 1988; Horgan, 2009) and crop varieties (Horgan, 2012). The consequences of such barriers to herbivory are important for crop managers, particularly those interested in protecting widely grown crops, such as cereals, from monophagous insects: for example, higher genetic diversity of crops at landscape levels could reduce between-field or regional movements of monophagous insects even without reliance on resistant varieties or resistance genes (Claridge and Den Hollander, 1982; Claridge et al. 1982; Horgan and Crisol, 2013).

Most herbivores form symbioses with intra- or extracellular prokaryotic and eukaryotic micro-organisms (Douglas, 1989). Endosymbionts may impart a range of benefits to their hosts – including protection against parasitoids (Ferrari et al. 2004) and pathogens (Chiel et al. 2007), and detoxification of chemicals (such as insecticides: Kikuchi et al. 2012; Pan et al. 2013). However, one of the most widely recognized functions of endosymbionts, particularly obligate intracellular symbionts, is the provisioning of essential amino acids and

vitamins to the insect host (Douglas, 1989; Ferrater et al. 2013; Hansen and Moran, 2014). In aphids, the obligate intracellular bacterial symbiont *Buchnera aphidicola* has been shown to increase aphid (*Acyrthosiphon pisum*) fitness by providing essential nutrients lacking in the host diet. Furthermore, secondary symbionts, some of which are also intracellular, have been implicated in aphid (*Acyrthosiphon pisum*) and whitefly (*Bemisia tabaci* [Gennadius]) host plant specificity (Chiel et al. 2007; Ferrari et al. 2004; Ferrari et al. 2007; Pan et al. 2013). However, a number of recent experimental studies with aphids suggest that feeding may not be the main benefit from these host-plant specific aphid-secondary symbiont associations (Leonardo, 2004; Ferrari et al. 2007, 2012; McLean et al. 2014). Nevertheless, in a convincing study of the role of symbionts in insect performance on host plants, Hosokawa et al. (2007) switched symbionts between two stinkbug species to attain a reversal in performance by the bugs on their respective hosts.

Planthoppers (Delphacidae) contain several bacterial and fungal symbionts (Ferrater et al. 2013). These include several species of yeast and yeast-like symbionts (YLS) (Ferrater et al. 2013). Among the best studied are YLS [Genebank accession no. AF267233.1], which occur in *Nilaparvata lugens* (Stål), *Sogatella furcifera* (Horváth) and *Laodelphax striatellus* (Fallén). YLS are members of the family Clavicipitaceae in the Pyrenomycetes class of fungi (Noda et al. 1995; Suh et al. 2001). These are obligate intracellular organisms that occur in specialized cells or 'mycetocytes'. In *L. striatellus*, these are located in the fat body cells of the abdomen. YLS abundance is highest in females at about the time of egg production (Noda, 1974) and the YLS are inherited maternally through the egg: Transovarial transmission of YLS has been described in detail by Cheng and Hou (2001) and Yukuhiro et al. (2014). YLS contribute to the nutritional requirements of planthoppers (Wetzel et al. 1992; Sasaki et al. 1996, Hongoh and Ishikawa, 1997; Ishikawa, 2003; Ganter, 2006). Because of their role in planthopper nutrition, it is not surprising that YLS have been suggested to play a

role in virulence adaptation by planthoppers to resistant rice varieties (Lu et al. 2004; Chen et al. 2011; Ferrater et al. 2013; Ferrater et al. 2015). For example, Lu et al. (2004) found YLS abundance in *N. lugens* to initially drop following switching from susceptible to resistant rice varieties and to later build-up over successive generations as the hoppers adapted to resistance. Furthermore, Chen et al. (2011) found that symbionts increased the fitness of *N. lugens* on several rice hosts but may become a 'drain' on the insects where host varieties are constant over several generations. Ferrater et al. (2015) found that YLS densities in *N. lugens* were determined by the rice variety on which *N. lugens* fed. In the same study, YLS densities declined after several generations on the same host variety suggesting that the symbionts may play a role in switching between plants, but were not as important in constant environments.

In the present study, we examine the role of YLS density on the ability of *N. lugens* to switch feeding between different rice varieties. *N. lugens* reared for several generations on each of five rice varieties (natal hosts) were switched to new varieties (exposed hosts) under normal YLS densities (symbiotic) and after reduction of YLS densities by heat treatment (aposymbiotic). We tested whether the symbionts had become a drain for the hoppers under constant feeding conditions (i.e., during successive generations of selection) and during switching, by comparing fitness of both aposymbiotic and symbiotic YLS that were either switched between rice varieties or maintained on the same varieties. If symbionts had become a drain for the planthoppers, we expected aposymbiotic hoppers to perform better than symbiotic hoppers, particularly where host varieties remained constant (i.e., no switching)(**Figure 1B,C**). We also examined the hypothesis that YLS densities play a role in host switching in *N. lugens*: we predicted that if YLS density affected the capacity of planthoppers to switch between hosts, any decline in fitness of aposymbiotic planthoppers compared to symbiotic hoppers would be greater when the planthoppers were switched to a new variety (exposed hosts) relative to their performance when switched between two plants

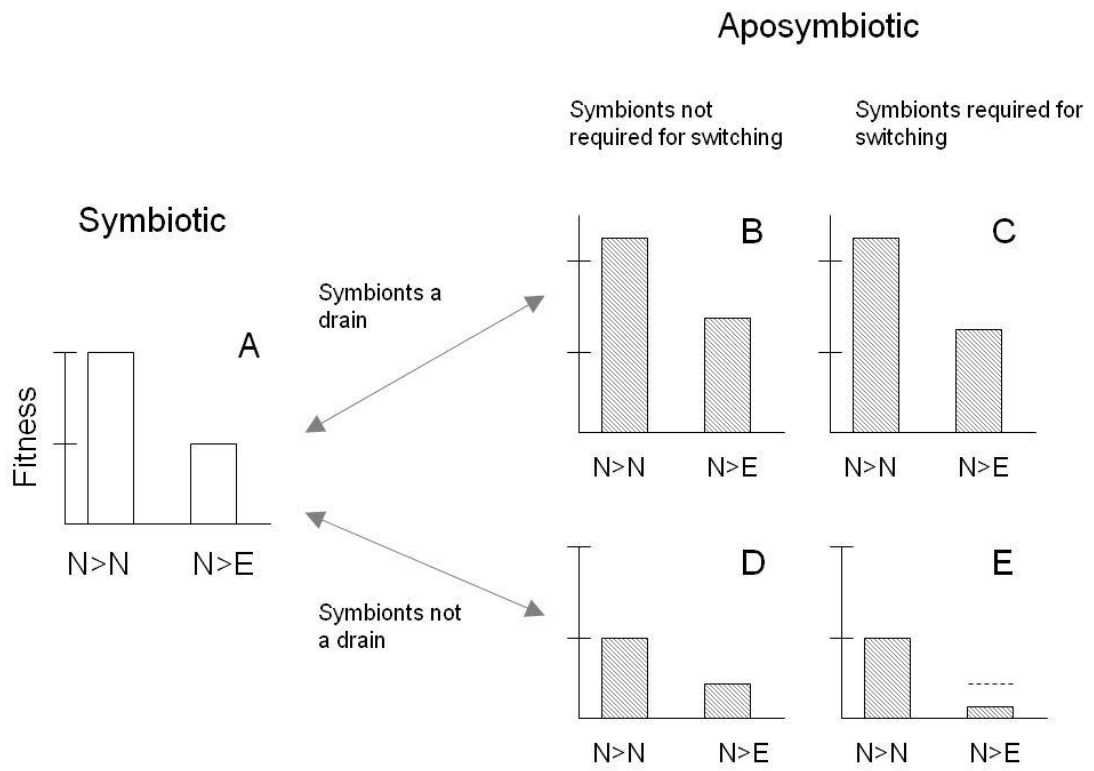

Figure 1. Predicted effects of removing YLS on planthopper fitness: Fitness of symbiotic hoppers reared on their natal host (natal to natal: N>N) declines when planthoppers are switched to a different host (natal to exposed: N>E)(A). If symbionts are a drain on hopper fitness, then removing symbionts increases fitness of both the hoppers that are moved between plants of their natal host (N>N) and from their natal host to a new host (N>E)(B,C); however, if symbionts are beneficial for the insect then removal will cause a fitness decline (D,E). Where symbionts are required for switching, fitness is disproportionately reduced in aposymbiotic planthoppers moved between varieties (N>E) compared to aposymbiotic planthoppers maintained on their natal host (N>N)(C,E).

of the same variety (natal hosts)(**Figure 1C,D**). We discuss our results in the context of symbiont-mediated host-plant adaptation by monophagous insects on resistant crops.

2.0 Materials and methods

2.1 Insect Populations

Brown planthopper, *Nilaparvata lugens* (Stål), populations were collected from Philippine rice paddies between September and November of 2009. The insects collected consisted of between 50-500 individuals from each of three geographical locations: Mindoro

[13.00° N, 121.08° E], Iloilo [10.72° N, 122.57° E], and South Cotabato - Mindanao [6.17° N, 125.00° E]). After five generations of building up populations on TN1, 500 gravid females of each of the three populations were collected and 100 each placed on five different rice varieties (see below) for selection (a total of 12 colonies). The 12 experimental colonies were continuously maintained on the different varieties during 20 generations of selection (approximately 24 months) during which time they were held in an insectary with a 12h:12h L:D regime and a constant temperature of ca. 26^{o}C. Further details on the selection procedure can be found in Ferrater et al. (2015). To avoid potential problems associated with inbreeding after 20 generations of rearing on the different varieties, those colonies reared on the same host variety but with different geographical origins were introgressed as a single colony on that same variety. This produced four colonies (henceforth the South-Central Philippines Experimental Colonies, SCPEC), one each reared on IR65482-4-136-2-2, IR62, IR22, or PTB33. To combine colonies, newly emerged adult males and females (100-200) were collected after generation 20 and placed on their designated host plants (according to the plant on which they were selected) in large aluminum wire mesh cages of 91.5 cm × 56.5 cm × 56.5 cm (H×L×W) in a greenhouse at the International Rice Research Institute (IRRI), Los Baños, Philippines. A further colony, collected at Laguna (Luzon, Philippines) was maintained for ca. 30 generations on Taichung Native 1 (TN1) and consistently maintained in large cages. This colony did not experience population decline during rearing and was therefore not introgressed with other similar colonies. This colony, henceforth (TN1-Laguna) was included in the experiments as a standard control. Temperatures in the greenhouse ranged between 26 and 45 ^{o}C over the course of the study, with natural daylight regimes (light ca. 6am to 6pm throughout the year). After >5 generations of mixing, the colonies were used in the experiments described below.

2.2 Plant materials

Five varieties were used in the experiments, with TN1 included as a standard susceptible variety often used in breeding studies (De Datta, 1981). The breeding line IR65482 contains the resistance gene *Bph10* derived from the wild rice *Oryza australiensis* Domin (Fujita et al. 2013). PTB33 is a traditional Indian variety considered among the most resistant rice varieties to Philippine populations of *N. lugens*. It has been used extensively in breeding programs aimed at increasing rice resistance to biotic stresses (Khush and Virk, 2005). The genetics of resistance in PTB33 is still not fully understood, but is likely governed by a number of major resistance genes (including *bph2* and *Bph3*: Angeles et al. 1986) and several minor genes (Santhanalakshmi et al. 2010). IR62 is a modern rice variety released by IRRI in 1984. The variety is highly resistant to *N. lugens* from Laguna. Its resistance has been attributed to the *Bph3* gene that was likely obtained from PTB33 (Khush and Virk, 2005; Peñalver Cruz et al. 2011). IR65482 and PTB33 are not commercially grown by rice farmers. IR62 is not commonly grown in the Philippines (<1% adoption among farmers - Peñalver Cruz et al. 2011), although it is popular among some farmers in Cambodia (P. Virk, personal communication). We used a further variety, IR22, as a susceptible control during the experiments. IR22 is thought to contain no major resistance genes (Brar et al. 2009).

All seeds of each of the five varieties, were acquired from IRRI: TN1 and PTB33 seeds were acquired through the International Network for Genetic Evaluation for Rice (INGER). The IR varieties (IR22, IR62 and IR65483) were acquired through the Plant Breeding, Genetics and Biotechnology Division. The seeds were planted and bulked-up in a screenhouse facility at IRRI.

2.3 Preference of *N. lugens* colonies for different varieties

Nymph settling choice bioassay: Five pots (7×11cm; H×D), each with a different variety of rice (from the five differentials), were placed in a rectangular mylar cage (29cm × 21.5cm × 56.5cm; H×L×W) in a circular configuration, without plants touching (**Figure 2**). Twenty newly-hatched nymphs from each of the five colonies were released into the separate cages. The nymphs were released to the centre of each cage and the number of nymphs settling on each variety was recorded after 5 days. There were five replicates for each colony.

Oviposition choice bioassay: Five pots (7×11cm; H×D), each with a different variety of rice (from the five different varieties) were placed in a rectangular mylar cage (29cm × 21.5cm × 56.5cm; H×L×W) in a circular configuration, without plants touching each other (**Figure 2**). Five gravid females from each of the ten colonies were released into the separate cages (a total of 25 cages each with 5 insects from a single colony). The females were released in the centre of each cage and the number settling on each variety was recorded after 3 days. The females were removed on day 3 after which the rice stems were cut at the base and the plants dissected under a stereomicroscope to count the eggs. There were five replicates for each colony.

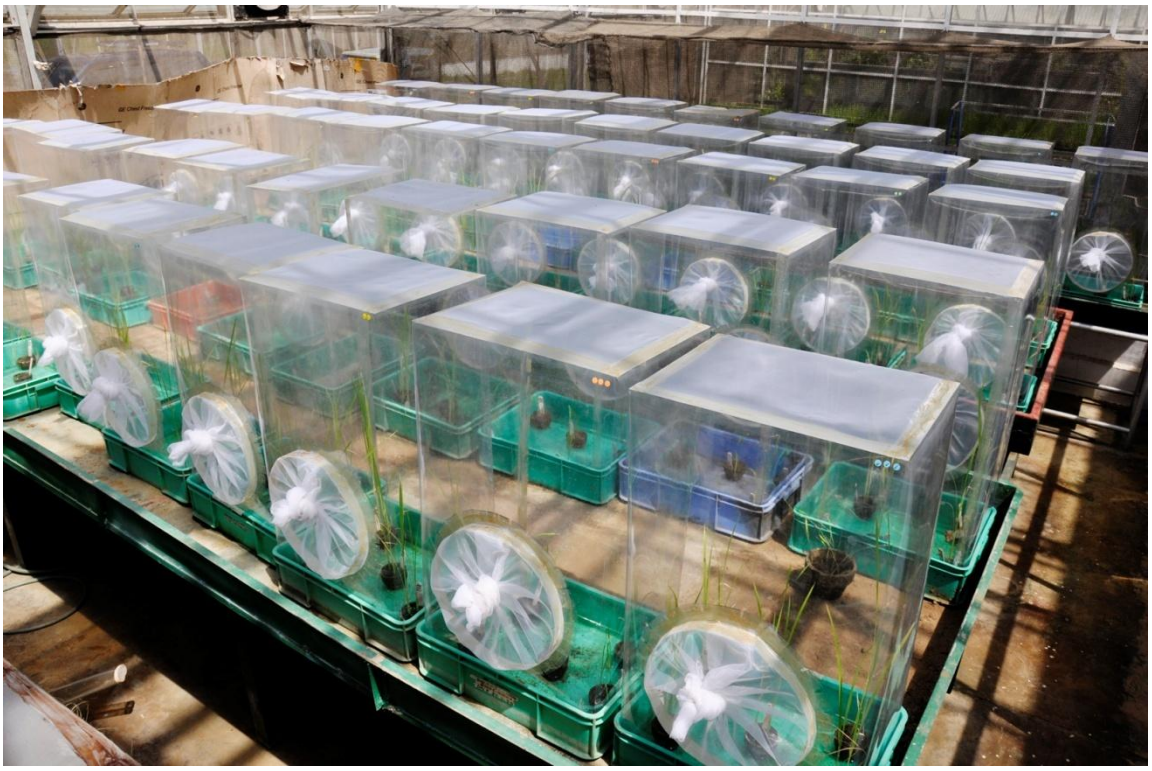

Figure 2. The choice bioassay set-up for nymphal and oviposition preferences of *Nilaparvata lugens.*

2.4 Role of YLS during host-plant switching

Newly-hatched nymphs from each of the 4 outbred SCPECs and the TN1-Laguna colony were placed on 20-day-old plants of each of the five different rice varieties. These were enclosed in plastic cages (61×10.5cm) over pots (7×11cm; H×D). After 48 hours at room temperature (26-28^{o}C), the plants were divided into two groups: One group was untreated to maintain normal, symbiotic planthoppers. The second group was moved to an environmental plant growth chamber set at 35^{o}C for 3 days to kill the symbionts and generate aposymbiotic planthoppers (Lu et al. 2004). After 3 days, the plants with aposymbiotic planthoppers were moved back to room temperature (again interspersing these plants with the first group) for the remaining 5 days. The planthoppers were exposed to the different varieties for a total of 10 days. After 10 days, the nymphs were removed from the plants. YLS densities were estimated from the nymphs following the method of Chen et al. (2011). The nymphs were weighed (wet weight) and homogenized in 500µl physiological saline solution

(0.9% NaCl). An aliquot of 10µl was transferred to a haemocytometer cell counter and suspended materials identified as YLS were counted under a compound microscope (40× magnification). YLS abundance was divided by the weight of the nymphs to indicate density.

2.5 Data analysis

Data from choice tests were ranked within colony and replication and analysed by univariate GLM. Tukey post-hoc comparisons were used to indicate homogeneous groups. Pearson correlation indicated a high correlation between nymphal settling preferences and both adult settling (N=50; C = 0.621; P < 0.001) and egg laying (N = 50; C = 0.581; P < 0.001). There was also a significant correlation between adult settling and egg laying (N = 50, C = 0.850, P < 0.001); therefore we only present data here from nymphal settling and egg laying.

We examined differences in the nymphal weights of planthoppers that were fed on their natal plant and planthoppers fed on a plant other than their natal host (henceforth exposed plant - i.e., representing a switch in host plant). Analyses were conducted separately for colonies reared on each natal host by univariate GLM. The model included two main factors – exposed/natal plant and symbiotic/aposymbiotic nymphs. We also examined proportional changes in symbiont densities for nymphs on exposed hosts relative to nymphs that were switched between plants of their natal host variety. Proportional changes may better represent the reproductive patterns of YLS. Proportional differences were analysed by univariate GLM with exposed plant and symbiotic/aposymbiotic nymphs as main factors.**3.0**

3.0 Results

3.1 Feeding and oviposition preferences in adapted *N. lugens*

Nymphs and adults selected for 25 generations on the susceptible rice variety TN1 demonstrated clear preferences for feeding/settling and egg-laying (respectively) on susceptible varieties in the choice tests. Nymphs and adults from the TN1-reared colonies preferred IR22 and TN1 for feeding and oviposition (**Table 1**; **Figure 3A,F**). Nymphs and adults reared on IR22 (susceptible) or on the resistant rice plants demonstrated no clear preferences for either feeding or oviposition on any of the five different varieties (**Table 1**; **Figure 3B-E, G-J**).

Table 1. F-values from ANOVA of settling and oviposition choice for SCPEC colonies reared on each of five natal varieties (see also **Figure 3**).

Natal Plant	Settling preference	Oviposition preference
	Exposed variety	Exposed variety
TN1	**17.059*** **	**4.533** **
IR22	0.349	0.774
IR65482	0.448	0.357
IR62	1.725	1.419
PTB33	1.525	1.889

1: Nominator DF = 4, denominator = 40; *** = P < 0.001, ** = P < 0.01

3.2 Responses by *N. lugens* during host-plant switching with normal and depleted YLS densities

Planthopper fitness (nymphal weight) and symbiont abundance varied considerably between colonies reared on different natal plants when these were transferred to a new variety (exposed plant)(**Figure 3**). Nymphs from the TN1 colony (susceptible variety) had lower weights when reared on plants other than their natal plant (TN1), even if the

exposed plant was considered susceptible (e.g., IR22)(**Table 2**; **Figure 4A**). Nymphs from the IR22 colony were significantly smaller when reared on PTB33 (**Table 2**; **Figure 4B**). We regard that for a nymph to be adapted to a resistant plant it should perform as well on that plant as on a susceptible variety such as TN1 or IR22; according to this criterion, the IR62, IR65482 and PTB33-reared colonies had apparently adapted to their natal hosts; IR62 and PTB33-reared colonies attained similar weights on all five host plants – indicating a broadening of host range as a result of selection (**Table 2**, **Figure 4D,E**); nymphs from the IR65482-reared colony attained very high weights on TN1 (suggesting physiological release) and performed as well on IR22 and PTB33 as on the natal host IR65482; however, they performed poorly on IR62 (**Table 2**; **Figure 4C**). Weight gain by nymphs from IR22-reared colonies was lowest on the resistant varieties and significantly lower on PTB33 than on the natal host (**Table 2**, **Figure 4B**). TN1-reared colonies performed well only on the natal host with significantly smaller nymphs on all other hosts including IR22 (**Figure 4A**).

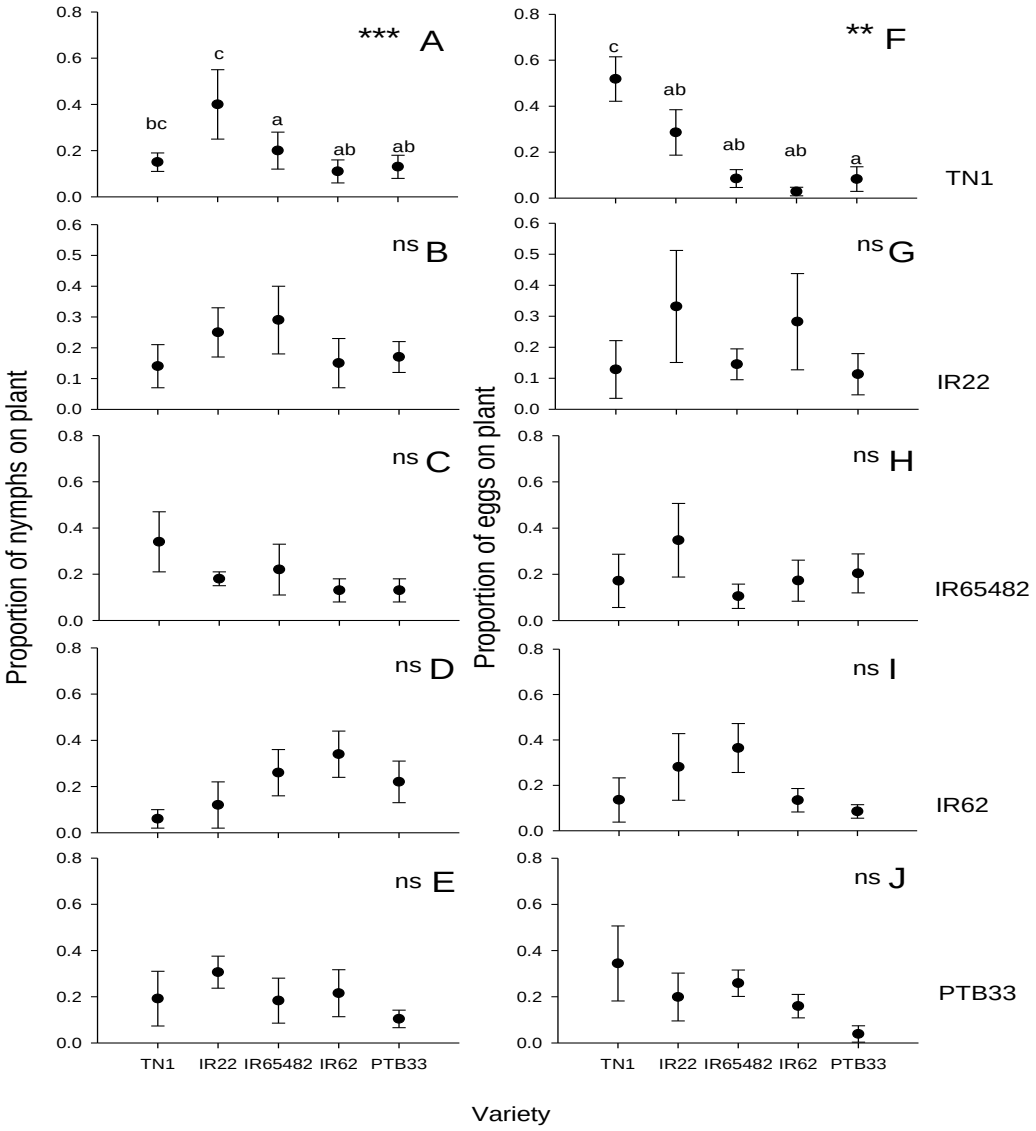

Figure 3. Settling preference (A-E) and oviposition preference (F-J) of *Nilaparvata lugens* nymphs and adults, respectively on five rice varieties in multiple choice experiment. Reactions of five colonies, were evaluated. The colonies were derived from populations reared for over 20 generations on TN1 (A, F), IR22 (B,G), IR65482 (C,H), IR62 (D,I) or PTB33 (E,J) as indicated on the right column. Results from univariate GLM for plant preferences are indicated as *** = $P < 0.001$, ** = $P < 0.01$ and ns = $P > 0.05$. Lowercase letters in A and F indicate homogenous groups (Tukey test: $P \geq 0.05$). Bars indicate standard errors (N = 5).

Aposymbiotic nymphs performed poorly on all host plants (**Table 2**, **Figure 4A-E**); however, the proportional weight changes on exposed plants relative to natal plants for symbiotic and aposymbiotic nymphs were generally similar indicating that the removal of symbionts (or heat treatment) influenced body weight but not the relative capacity of nymphs to feed on the different plants (**Table 2**; **Figure 4F,G,H,J**). There was however one exception: aposymbiotic IR62-reared hoppers had lower weight gain on TN1 compared to symbiotic hoppers from the same colony (IR62-reared)(**Figure 4I**). In this case, the proportional difference in symbiont densities between insects on the exposed plants (TN1) relative to their natal plants (IR62)(i.e., [density on exposed/density of natal]-1) were not affected by the identity of the exposed plants (**Table 2**; **Figure 4I**). Furthermore, proportional differences in symbiont densities were similar between symbiotic and aposymbiotic hoppers from IR22-, IR65482- and PTB33-reared colonies (Fig. 3L,M,O), but were significantly lower for aposymbiotic hoppers from the TN1-reared and IR62-reared colonies when these were changed from their natal variety to a different plant variety (**Table 2**; **Figure 4K,N**).

Table 2.F-values for the effects of exposed variety and yeast-like symbionts (YLS) (symbiotic or aposymbiotic) on the nymph weight, weight response and YLS density response of *Nilaparvata lugens* from SCPEC colonies reared on each of five natal varieties (see also **Figure 4**).

Natal variety	Nymph weight[1,4]		Nymph weight response[2,4]		YLS density response[3,4]	
	Exposed variety	YLS	Exposed variety	YLS	Exposed variety	YLS
TN1	**23.892****	**23.117****	**11.483*****	1.043	1.727	**10.293*****
IR22	**22.553****	**174.057*****	**3.412***	0.004	0.584	0.010
IR65482	**17.607****	**122.958*****	**16.755*****	0.680	1.847	0.008
IR62	0.774	**48.824*****	0.878	**5.468***	2.776	**11.060****
PTB33	6.258	**61.378*****	2.474	0.417	1.757	2.582

1: DF = nominator exposed variety = 4, YLS = 1, denominator = IR65482, PTB33 = 50, TN1, IR22 = 49; IR62 = 47

2: DF = nominator exposed variety = 3, YLS = 1, denominator = 40

3: DF = nominator exposed variety = 3, YLS = 1, denominator = 40

4: *** = $P < 0.001$, ** = $P < 0.005$, * = $P < 0.05$; all interactions are non significant, $0.112 \leq F \leq 2.494$

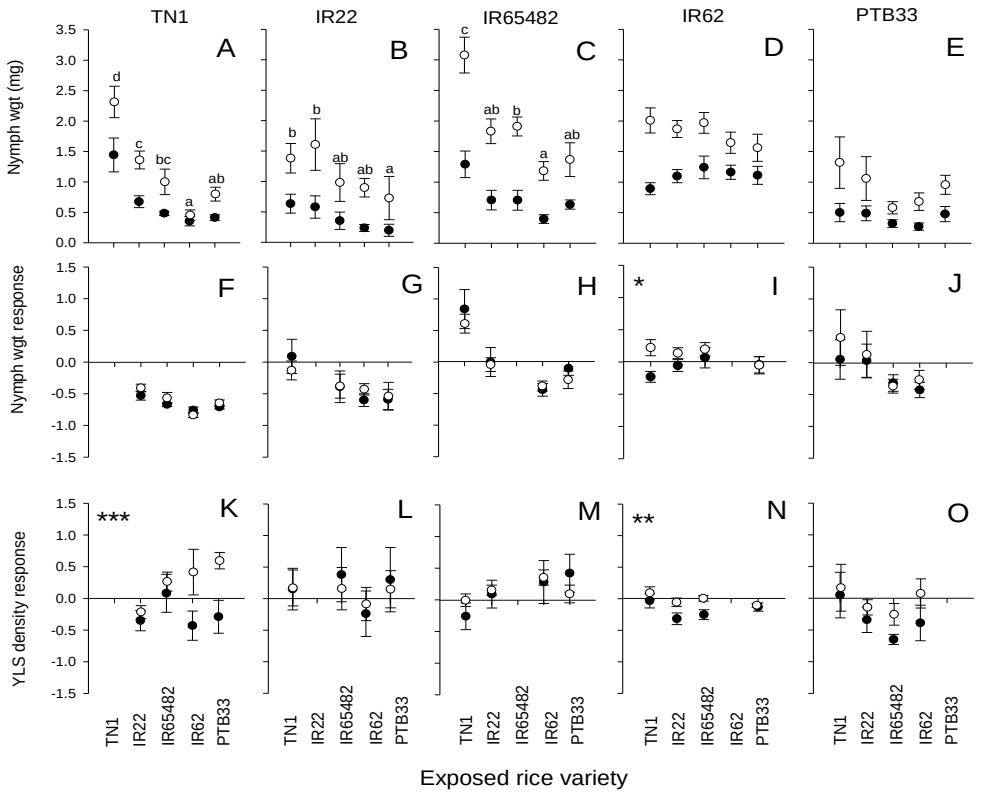

Figure 4. Nymph weight (A-E), nymph weight response ([weight on exposed variety/weight on natal variety]-1)(F-J), and proportional yeast-like symbiont density ([density on exposed variety/density on natal variety]-1)(K-O) on five exposed varieties for SCPEC outbred colonies derived from colonies reared on TN1 (A,F,K), IR22 (B,G,L), IR65482 (C,H,M), IR62 (D,I,N) and PTB33 (E,J,O). Open symbols indicate symbiotic nymphs, solid symbols indicate aposymbiotic nymphs. Bars indicate standard errors (N = 5). The zero lines in F-O indicate responses where natal and exposed varieties are the same (i.e., F: TN1 = natal variety and zero line is response on TN1 exposed variety). Lower case letters in A-C indicate homogenous groups (Tukey test: $P \geq 0.05$). * = $P < 0.05$, ** = $P < 0.01$, and *** = $P < 0.001$ for differences between symbiotic and aposymbiotic nymphs.

4.0. Discussion

There is now considerable evidence to suggest that prokaryotic and eukaryotic endosymbionts play a role in determining herbivore preferences for, and fitness on, different host plant species (Barbosa et al. 1991; Clark et al. 2010; Frago et al. 2012; Ferrater et al. 2013; Hansen and Moran 2014). Much of this evidence is recent and has come from both descriptive and manipulative experiments with aphids (Ferrari et al. 2004, 2007, 2012; Tsuchida et al. 2004, 2011; McLean et al. 2011) and stinkbugs (Hosokawa et al. 2007). However, the studies that most strongly support the role for symbionts in herbivore selection of host plants have mainly examined the performance of different insect species feeding on distinct host plant species (Hosokawa et al. 2007) or preferences for feeding by a single herbivore species on different host plant species (Tsuchida et al. 2004, 2011). In contrast, the present study is one of very few to examine the role of symbionts in defining virulence and fitness responses in a monophagous species feeding on different varieties of a single host plant species (Lu et al. 2004; Chen et al. 2011). Many of the most problematic herbivore pests of agricultural crops are monophagous or at least oligophagous (i.e., Hessian fly, *Mayetiola destructor* (Say), Asian rice gall midge, *Orseolia oryzae* (Wood-Mason), Russian wheat aphid, *Diuraphis noxia* (Kurdjumov), and *N. lugens*) and have demonstrated virulence adaptation to varieties bred for resistance (Fujita el al. 2013; Lu et al. 2013; Tolmay et al. 2013; Garces-Carrera et al. 2014). The role for symbionts in such adaptations (representing relatively minor shifts in host plant genetics compared to interspecific host plant shifts) is poorly understood. This study, which included a range of rice varieties that varied from susceptible to resistant against *N. lugens*, suggests that whereas YLS are essential for normal planthopper development, they do not play any major role during host-plant switching. The general trends noted when switching symbiotic and aposymbiotic nymphs between rice hosts

(natal and exposed) in our study suggest that in most cases YLS densities (and, therefore, functional capacity) do not determine variety-related changes in fitness of the insect host.

It is well established that obligate intracellular symbionts play an essential role in providing nutrients for their insect hosts (Tsuchida et al. 2004, 2011; Ferrater et al. 2013; Hansen and Moran, 2014). The experimental removal or density reduction of symbionts results in a series of abnormalities in host development as demonstrated for stinkbugs (Hosokawa et al. 2007), planthoppers (Ferrater et al. 2013) and aphids (Hansen and Moran, 2014). In a classic experiment, Hosokawa et al. (2007) demonstrated the role of obligate symbiotic bacteria in determining performance by stinkbugs: these authors exchanged bacteria between *Megacopta punctatissima* (Montandon), which performs well on crop legumes, and a closely related non-pest congeneric, *Megacopta cribraria* (Fabricius), which performs poorly on the same plants. Once the symbionts had been exchanged, the two insect species demonstrated a marked reversal in their performance (hatching rates) on the crop legumes. Despite such studies, there is still little convincing evidence of a role for symbionts in determining host plant specificity in a single herbivore species (i.e., aphids [Ferrari et al. 2004, 2007, 2012; Mclean et al. 2011] and planthoppers [Tang et al. 2010]). When tested using manipulative experiments, the widely documented associations between the secondary symbionts of aphids and their preferred host plant species have not generally indicated a role for the symbionts in determining aphid 'biotypes' (i.e., populations specialized for feeding on a particular host plant species or variety)(Ferrari et al. 2004, 2007, 2012; Mclean et al. 2011). A similar association between bacterial symbionts and *N. lugens* populations that were selected over several generations on resistant rice varieties has been demonstrated by Tang et al. (2010), but has not received further attention. In contrast to bacterial symbionts, the YLS of planthoppers have been relatively well researched (Ferrater et al. 2013). Reducing YLS abundance through heat treatment results in protein deficiencies in planthopper eggs (Lee and

Hou, 1987) and reduced protein content in nymphs (Wilkinson and Ishikawa, 2001) leading to retarded growth of nymphs and death of fifth instars during ecdysis (Chen et al. 1981b). Heat treatment of hoppers in the present study reduced YLS densities by between 80 and 88% and was associated with lower weight gain of nymphs on all rice varieties (**Figure 4**). YLS densities have been shown to vary among planthoppers selectively reared on different rice plants (Lu et al. 2004; Ferrater et al. 2013). This suggests that there is, at least, a planthopper-mediated response by symbionts to the chemistry and nutritional quality of the host variety. YLS densities have been shown to increase over time in the study by Lu et al. (2004), but remained relatively constant – albeit with a slight increase in density at the start of selection - in the study by Ferrater et al. (2015). Meanwhile, Chen et al. (2011) suggested that after several generations in a constant environment (including on a single host variety), YLS may become a drain to the planthoppers. This suggestion was supported by Ferrater et al. (2015), who noted declining densities of YLS as selection progressed on several rice varieties. However, in the present study, where YLS were reduced through heat treatment, we found no advantage for nymphs when fed either on their natal host or when switched to a new host variety. Furthermore, for 19 of 20 cases where we switched hoppers to feed on a new host, we found that the relative fitness of hoppers on the exposed varieties was very similar or identical for aposymbiotic and symbiotic planthoppers. This suggests that YLS density plays little role during planthopper switching. This is further supported by similarities in the abilities of planthoppers reared continuously on IR22, that have naturally low YLS densities, and on PTB33, with high densities (Ferrater et al. 2015), to switch between host varieties.

YLS are known to contribute to the nitrogen requirements of planthoppers (Douglas 1989). Under high nitrogen diets, planthoppers store uric acid in their tissues. However, under nitrogen stress, uric acid is converted into compounds of nutritional value by YLS through the action of symbiont uricase (Sasaki et al. 1996; Hongoh and Ishikawa, 1997;

Ishikawa, 2003; Ganter, 2006). YLS are also involved in sterol metabolism (Noda and Saito 1979; Eya et al. 1989; Wetzel et al. 1992). Switching by insect herbivores between plants is a common phenomenon and can occur where the plants in one patch are depleted or become otherwise unsuitable or unavailable (as during rice harvest). Planthoppers may spend as many as 3-4 generations on a single host plant individual or in a single rice field before they must switch fields or varieties. For monophagous insects like *N. lugens*, switching between hosts often results in a decline in fitness that may be observed during several generations. This occurs even where the hoppers are switched between two relatively susceptible hosts (Horgan, 2012) and, as an example, occurred in the present study on switching nymphs from TN1 (susceptible) to IR22 (susceptible). Reduced feeding efficiency on a new host could lead to temporarily poor nutrient acquisition from the host plant. It is intuitive that at this stage, YLS might compensate for depleted nutrient availability by converting stored uric acid. The present study examines the effects of only one aspect of YLS - density - on fitness and function of the planthopper host. It is now apparent that several YLS occur in planthoppers, and at least one of these occurs in mycetocytes located in abdominal fat bodies (Ferrater et al. 2013). The role and efficiency of the YLS community may be related to community composition (relative abundance and diversity of different YLS species) or overall density (which may or may not affect community evenness). We found little evidence of a role for YLS densities during switching; however, this does not suggest that the YLS have no function during such an event. They may play a role that is not density dependent, and apparently, as indicated in the results from our study, the magnitude of the role may be variety dependent. However, it is clear that the role of YLS density during switching does not depend on whether the natal or exposed hosts are resistant or susceptible. Fitness reductions were observed upon moving aposymbiotic planthoppers between two other

resistant hosts (IR65482, IR62) and the susceptible standard TN1; however, in these cases the decline in fitness that resulted from heat-treatment was not statistically significant.

We used heat treatment to produce aposymbiotic planthoppers. Heat-treated planthoppers demonstrate several physiological and developmental deficiencies that have been associated with low YLS abundance (Vega and Dowd, 2005; Ferrater et al. 2013). However, heat treatment may have unknown effects on the composition or abundance of bacterial symbionts, on different YLS species or on the planthoppers themselves. For example, Piyaphongkul et al. (2012) suggest that the critical maximum temperature for brown planthopper nymphs is 34.9^{o}C – the same temperature used to produce aposymbiotic nymphs. Whereas these observations suggest that reduced planthopper fitness as a result of heat treatment may not have a direct mechanistic link between the symbionts and the planthoppers feeding on different hosts, our observation of generally similar responses among aposymbiotic (heat-treated) and symbiotic (untreated) hoppers when switched between host varieties (**Figure 4F-J**) suggest that the high temperatures that we used (35°C) did not affect the aspects of planthopper fitness in which we were interested (i.e., traits related to feeding).

The emerging picture from research into the role of symbionts in planthopper feeding and host plant specificity shares many features with results from research on the symbionts of aphids. Aphids, like planthoppers (Ferrater et al. 2013), have been shown to harbor a diversity of microorganisms that include intracellular and extracellular primary and secondary symbionts (Ferrari et al. 2004; 2007; 2012; Tsuchida et al. 2004, 2011). For aphids, these symbionts provide a range of diverse benefits to the host insect (Hansen and Moran 2014). In planthoppers, the role of symbionts in host nutrition and virulence adaptation has received most attention (Lu et al. 2004; Tang et al. 2010; Chen et al. 2011; Ferrater et al. 2013). The obligate bacterial symbiont *B. aphidicola* is essential for aphid

development (Hansen and Moran, 2014) in the same way that YLS are essential for development in planthoppers (Ferrater et al. 2015). Host-plant specific associations between aphid host races and secondary bacterial symbionts have been noted (Ferrari et al. 2004, 2007, 2011). Evidence suggests that such specificity might also exist among planthoppers reared on different host varieties (Tang et al. 2010). However, in both cases, there is no evidence that these secondary symbionts mediate host plant specificity for their respective insect hosts (e.g., aphids: Ferrari et al. 2004, 2007, 2011, McLean et al. 2011). To our knowledge, obligate symbionts have not yet been associated with virulence adaptation – although YLS densities do change during switching and virulence adaptation in planthoppers (Ferrater et al. 2015). Noted density responses in YLS during selection and switching imply planthopper-mediated responses by the symbionts to the host varieties (some of which are highly resistant to planthoppers) but not symbiont density mediated adaptation or symbiont density related facilitation of switching. Furthermore, YLS were not observed in this study to represent a drain on insect fitness as suggested in one previous study (Chen et al. 2011). Future studies will need to examine aspects other than changing densities of obligate symbionts during adaptation and switching, and should employ techniques other than heat treatment. Nevertheless, given the intuitive relation between density and magnitude of function for YLS communities, our study suggests that YLS are unlikely to have any significance for the ability of brown planthoppers to switch between host varieties.

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CHAPTER 5

Can virulence be acquired by *Nilaparvata lugens* from conspecifics at shared feeding sites? A test of facilitated feeding on a resistant rice variety following infestation by virulent planthoppers

Jedeliza B. Ferrater and Finbarr G. Horgan

Abstract

This study examines the possibility of horizontal and vertical transmission of virulence promoting factors between individuals from brown planthopper (*Nilaparvata lugens* Stål) populations with distinct feeding abilities when their populations share the same feeding sites (Virulence Acquisition Hypothesis). We created optimal conditions for intraspecific interactions between planthoppers from populations with different feeding histories (virulent and avirulent) on the same rice plants. We then introduced avirulent planthoppers to the same plants. We noted that planthoppers attained highest weights on those plants on which virulent (IR62 resistance-adapted) planthoppers had previously fed, compared to plants on which avirulent planthoppers or no planthoppers had previously fed. Therefore, feeding by the virulent planthoppers facilitated subsequent planthopper feeding on the same plant. We also tested the ability of avirulent planthoppers to feed on the resistant variety IR62 and the susceptible variety TN1 after interacting with virulent hoppers on a third host (Triveni). Feeding on IR62 or TN1 was improved by sharing feeding sites with virulent planthoppers in one of two runs of the experiment, indicating that facilitation may be mediated through the insect. We then tested progeny of the same planthopper populations for potential improvements in feeding ability on the resistant variety IR62 after their parents had shared feeding sites with IR62-virulent planthoppers. Progeny attained similar weights and excreted similar amounts of honeydew irrespective of the feeding history of their parent (i.e., whether these had shared feeding sites with virulent planthoppers) in the first run of the experiment, but showed improved feeding on IR62 in the second run. These results indicate that feeding by mixed virulent-avirulent populations may accelerate adaptation by *N. lugens* to resistant rice varieties.

Submitted for publication.

1.0 Introduction

Although many plants produce defensive compounds in response to injury by insect feeding, herbivorous insects can adapt to these plant responses through altered feeding and behavior (Habibi et al. 2001; Wu and Baldwin, 2010). In order to feed, sucking insects such as aphids and planthoppers use their stylets to mechanically penetrate plant tissues (Walling, 2008). During stylet probing and upon sap feeding, these insects introduce oral secretions (OS) into the plant. Insect salivary secretions contain digestive enzymes but may also contain elicitors that induce plant defenses or effectors that inhibit the defenses (Miles, 1999; De Vos and Jander, 2008; Hogenhout and Bos, 2011; Bonventure, 2012). The salivary components move and travel through the vascular systems and potentially circulate throughout the plant tissues (Madhusudhan and Miles, 1998). Apart from OS-derived proteins, herbivores may secrete microorganisms along with their saliva that influence plant-insect interactions (Maischak et al. 2007; Chung et al. 2013). Many types of bacteria are transmitted by sucking insects that feed on plant sap and are capable of systemic movement throughout the plant; furthermore, depending on the abundance of microbes, these can alter observed levels of insect virulence (Purcell and Hopkins, 1996).

The brown planthopper, *Nilaparvata lugens* (Stål), is one of the most destructive insect pests of rice (O*ryza sativa* L.) causing substantial losses in South and East Asia (Bottrell and Schoenly, 2012). The cultivation of resistant rice varieties is an important component of *N. lugens* management. A few recent studies have investigated the components of *N. lugens* saliva and compared these components among virulent (adapted to plants carrying resistance genes, i.e, cultivar Mudgo) and avirulent (reared on rice varieties such as Taichung Native 1 (TN1) that have no resistance genes) planthoppers. These recent studies have applied novel approaches to compare saliva from the insects (proteomics: Konishi et al. 2009; transcriptomics: Ji et al. 2013; and immunodetection analyses: Petrova and Smith

2014). Recently, Wang et al. (2008) and Tang et al. (2010) observed large numbers of bacteria-like organisms (BLOs) in the salivary sheaths of *N. lugens* and at planthopper feeding sites in rice. The function of these BLOs in the saliva is still unknown and information on whether they are naturally associated with feeding is still unavailable. Together, these studies indicate that under conditions of high planthopper density, during planthopper outbreaks, feeding sites can become a focal point for contamination (i.e., OS and BLOs) between planthoppers with different feeding histories. Indeed, during planthopper outbreaks rice plants are often partially coated with a mix of planthopper honeydew and other excreta, fungi and plant exudates (Fujita et al. 2013).

Planthopper virulence adaptation is usually regarded as a gradual shift in the ability of planthoppers to feed and oviposit on resistant varieties following continuous selection on an abundant, resistant host (Alam and Cohen, 1998; Ferrater et al. 2013, 2015). Few studies have examined the underlying mechanisms of adaptation, but many researchers have predicted detectible shifts in genetically determined virulence (Tanaka, 1999; Jing et al. 2014; Kobayashi et al. 2014; Yu et al. 2014). We hypothesize that virulence may also be acquired indirectly through virulence-promoting factors (OS and BLOs) that are passed between insects that feed on the same plant. We call this hypothesis the Virulence Acquisition Hypothesis (VAH) drawing attention to potential non-genetic mechanisms that enhance herbivore feeding on resistant plants. The VAH holds that planthoppers may gain virulence against resistant rice varieties by acquiring virulence factors indirectly through host plants on which virulent planthoppers contemporaneously feed or had previously fed. We created optimal conditions for feeding-associated contamination of a tolerant host plant (cv. Triveni) by a planthopper population with virulence against a resistant variety (IR62, which possesses the *Bph3* gene). We then introduced avirulent planthoppers to the same plants where they might acquire potential virulence factors. Subsequently, we tested the avirulent planthopper

populations for improvements in fitness on the same and novel varieties, including the resistant variety IR62. An increase in fitness observed among the avirulent planthopper strain would support the VAH.

2.0 Materials and Methods

2.1 Plant materials

For the purpose of these experiments, *N. lugens*-resistant, susceptible and tolerant rice varieties were required (**Table 1**). We selected IR62 as a modern variety with recognized high resistance to Philippine populations of *N. lugens* (Peñalver Cruz et al. 2011). Resistance in IR62 rice is associated with the *Bph3* resistance gene present in traditional South Asian varieties such as Rathu Heenati and PTB33 (Fujita et al. 2013). The gene in IR62 was likely acquired through rice variety PTB33 (although IR62 has a complex pedigree: Khush and Virk, 2005). Triveni, a traditional Indian rice variety, is recognized as tolerant to *N. lugens* (Ho et al. 1982). Tolerance is the ability to withstand or compensate for attack while maintaining biomass growth and grain yield. TN1 is commonly used as a standard susceptible control in studies of rice resistance conducted in South and South East Asia and possesses no known resistance to Asian *N. lugens* (De Datta, 1981). Variety IR22 was used as an alternative susceptible variety and is often the preferred host of *N. lugens* (Ferrater et al. 2015). Though not used directly in the experiments, we used *N. lugens* colonies that had been reared on this variety for several generations (**Table 1**).

Table 1. Plant and insect materials (*Nilaparvata lugens* colonies) used in experiments.

Rice accession	Origin	*N. lugens* resistance genes	Purpose in this study	Associated selected colonies
Triveni	Traditional Indian variety[1]	None	Highly tolerant, used to permit high *N. lugens* infestations without killing the plant	None
IR62	Variety released by IRRI in 1984 – PTB33 used as resistance donor[2]	*Bph3*	Highly resistant to *N. lugens*[4]	IR62-SCPEC (30 generations of continuous rearing on IR62)[4,5]
Taichung Native 1 (TN1)	Variety released in 1960 in Taiwan; used as a standard susceptible check at IRRI[3]	None	Susceptible check and host for avirulent *N. lugens*	TN1-Laguna (30 generations of continuous rearing on TN1)[4]
IR22	Variety released by IRRI in 1969; Highly susceptible to *N. lugens*[2]	None	Alternative susceptible check and host for avirulent *N. lugens*	IR22-SCPEC (30 generations of continuous rearing on IR22)[4,5,6]

1: Ho et al. 1982; 2: Khush and Virk, 2005; 3: De Datta, 1981; 4: Ferrater et al. 2015[Chapter 3]); 5: South-Central Philippines Experimental Colonies [SCPEC](see text); 6: IR22-reared colony is used as a test colony to examine the effects of feeding on plants that were previously attacked by adapted (IR62) and non-adapted (TN1) planthoppers.

Seeds of Triveni were acquired from the International Network for Genetic Evaluation of Rice (INGER) at the International Rice Research Institute (IRRI) in the Philippines. Seeds of IR22, IR62 and TN1 were acquired from the Plant Breeding, Genetics and Biotechnology (PBGB) Division at IRRI. Prior to the experiments, the seeds were incubated for 72 hours at 60^{o}C to break dormancy. After incubation, seeds were soaked in water for 24 hours and then transferred to Petri dishes lined with moistened absorptive paper for 72 hours. The Petri dishes were incubated in a dark room at ambient temperature (28^{o}C). Germinated seedlings were transplanted to soil in clay pots (10×12 cm; H×D). All

experiments were conducted in a greenhouse at IRRI. Temperatures in the greenhouse ranged between 28 and 37^{o}C over the course of the study under the natural daylight regime (light ca. 6am to 6pm throughout the year).

2.2 Insect Populations

The brown planthopper (*N. lugens*) (Delphacidae: Hemiptera) is a largely monophagous specialist on rice. The planthopper is widely distributed throughout tropical Asia and the South Pacific, and also occurs in Australia (Bottrell and Schoenly, 2012). Although outbreaks can occur naturally in North East Asia (China, Japan and Korea), in the tropics *N. lugens* is regarded as a Green Revolution pest with outbreaks largely associated with modern rice varieties grown under intensified conditions with excessive use of fertilizers and resurgence-causing insecticides (Bottrell and Schoenly, 2012; Horgan and Crisol, 2013). For decades the principal management response to *N. lugens* has been to develop resistant rice varieties. Currently some 80 'hopper' resistance genes have been identified of which 34 demonstrate moderate to high resistance against *N. lugens* (Fujita et al. 2013). However, several *N. lugens* populations are now virulent against a range of formerly resistant varieties (Myint et al. 2009b; Fujita et al. 2013; Ferrater et al. 2013).

We used three *N. lugens* colonies for these experiments: Adapted to IR62 [*Bph3*], a non-adapted colony reared on TN1, and a second non-adapted colony reared on IR22. The IR62-adapted *N. lugens* colony was developed using populations originally collected from Philippine rice paddies between September and November of 2009. The insects collected consisted of between 50 and 500 individuals from each of three geographical locations: Mindoro [13.00° N, 121.08° E], Iloilo [10.72° N, 122.57° E], and South Cotabato - Mindanao [6.17° N, 125.00° E]). After five generations of building up populations, 200 gravid females of each of the three populations were collected and 100 each placed on the two rice varieties

for selection (a total of six colonies). The six experimental colonies were continuously maintained on the different varieties during 20 generations of selection (approximately 24 months) during which time they were held in an insectary with a 12h:12h L:D regime and a constant temperature of ca. 26^{o}C. Further details on the selection procedure can be found in Ferrater et al. (Chapter 6). To avoid problems associated with inbreeding after 20 generations of rearing on each of the differential varieties, the six colonies were admixed as two colonies (henceforth the South-Central Philippines Experimental Colonies, SCPEC) producing a single colony reared on IR62 or IR22. To combine colonies, newly emerged adult males and females (100 to 200 individuals) were collected after generation 20 and placed on their designated host plants (according to the plant on which they had been selected) in large aluminum wire mesh cages of 91.5×56.5×56.5 cm (H×L×W) in the greenhouse. The third colony, collected at Laguna (Luzon, Philippines) was maintained in large cages for ca. 30 generations on TN1. This population did not experience population decline during rearing and was therefore not introgressed with other, similar colonies (**Table 1**).

2.3 Experiment 1: Responses by *N. lugens* to prior feeding by conspecifics on a single host plant

Twenty four 20-day-old Triveni plants were each enclosed in cylindrical mylar cages (61 ×10.5cm: H×D) (**Figure 1A**), each with a mesh side window and mesh top for ventilation. The plants were divided into three groups: Plants in the first group (8 pots) were each infested with 10 unmated IR62-SCPEC *N. lugens* females. Plants in the second group (8 pots) were each infested with 10 unmated *N. lugens* females from the TN1- Laguna colony. Plants in the third

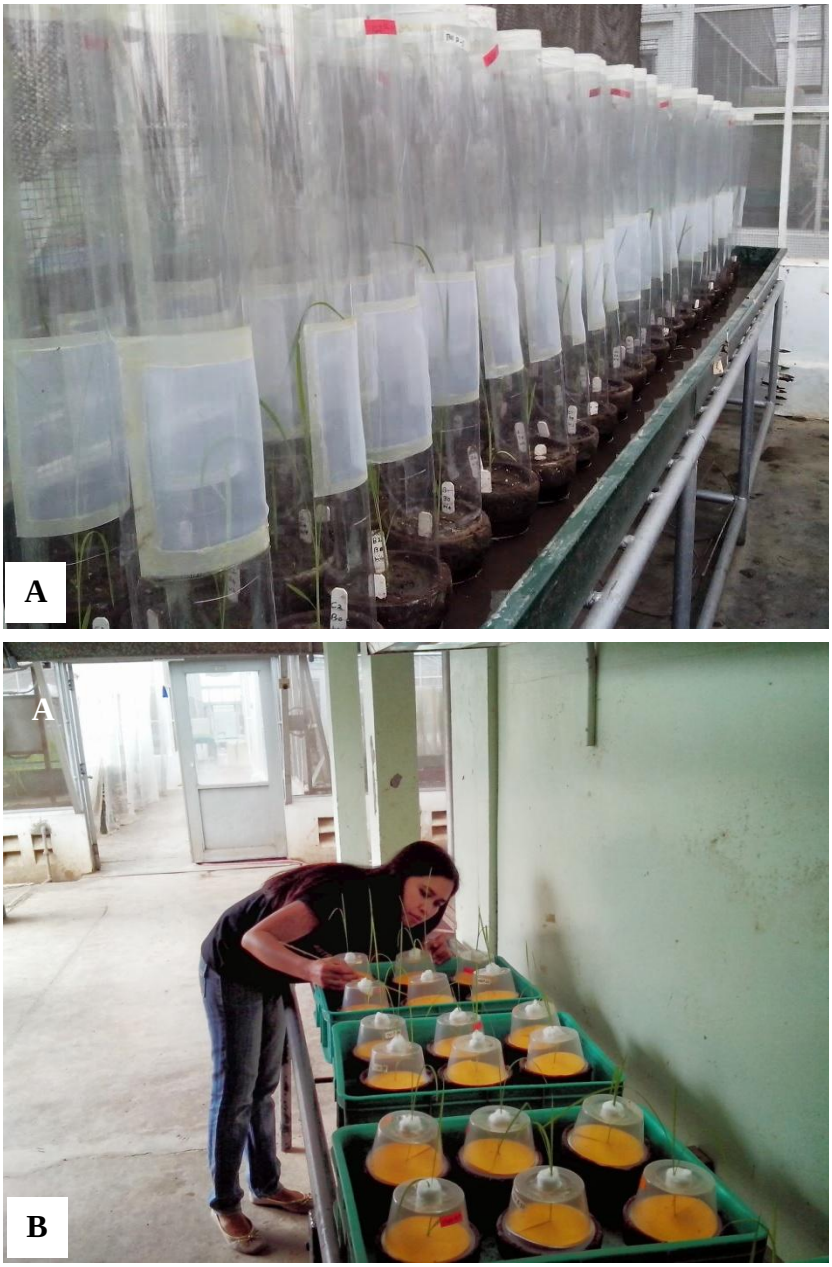

Figure 1. Experimental set-up for the *Nilaparvata lugens* on a tolerant variety, Triveni: A) Experiment 1, Responses by *N. lugens* to prior feeding by conspecifics on a single host plant B) Experiment 2, Feeding response on IR62 (resistant) and TN1 (susceptible) rice varieties by progeny of avirulent *N. lugens.*

group (8 pots) were not exposed to *N. lugens* (uninfested controls). The plants were laid out in a completely randomized design on a bench in the greenhouse. The planthoppers were allowed to feed on the plants for 4 days. After 4 days all the planthoppers were removed and discarded. All plants were reinfested with two newly emerged, unmated adult female *N. lugens* from the IR22-SCPEC. The planthoppers had been starved for 1 hour prior to infestation. Honeydew excreted by these insects was monitored using the method of Pathak and Heinrichs (1982) using specially prepared plastic chambers that restricted the hoppers to within 5cm of the base of the plants. The chambers were placed on top of filter paper, neatly fitted around the plant shoot. The filter papers had been treated with bromocresol green. Bromocresol green indicates the nature of the honeydew as coming from the phloem (basic reaction indicated by blue-rimmed spots) or xylem (acidic reaction indicated by white spots) (Yesuraja and Mariappan, 1991). The area of excreted honeydew spots on the bromocresol-treated filter paper was measured using Image J software version 1.48 (National Institutes of Health, USA). The insects used in the honeydew feeding test were collected, oven-dried at 60ºC for 3 days, and weighed. All honeydew results were standardized by the weight of the insects used in the bioassay.

Immediately after the honeydew test, plants were infested with ten neonate *N. lugens* (IR22-SCPEC). The nymphs were allowed to feed and develop on the plants for 15 days. After 15 days, the nymphs were collected, oven-dried at 60ºC for 3 days, cleaned of debris and weighed. The plants were then placed in paper envelopes separating the above (shoots) and below ground (root) parts. Rice plants were oven-dried at 60ºC for 7 days and weighed.

2.4 Experiment 2: Feeding response on IR62 and TN1 rice varieties by progeny of avirulent *N. lugens* from plants previously attacked by virulent conspecifics

An experiment to examine possible vertical transmission of virulence from parents (avirulent) to progeny after the parents had shared feeding sites with virulent planthoppers was conducted in the greenhouse at IRRI. The experiment was repeated two times (henceforth indicated as 'run A' and 'run B') with minor changes to the protocol as indicated in **Table 2**.

Thirty 20-day-old Triveni plants were each enclosed in cylindrical mylar cages (Run A: 45×5cm H×D; Run B: 61× 10.5cm H×D), each with a mesh side window and mesh top for ventilation. The plants were divided into three groups: plants in the first group (10 pots) were each infested with 10 unmated IR62-SCPEC *N. lugens* females. Plants in the second group (10 pots) were each infested with 10 unmated TN1- Laguna *N. lugens*. Plants in the third group (10 pots) were not exposed to *N. lugens* (uninfested-controls) (**Figure 2**: Step 1). The plants were laid out in a completely randomized design on a bench in the greenhouse. The insects were allowed to feed on the plants for a few days (Run A: 2 days; Run B: 4 days). After this time the insects were removed using a hand-held pooter and discarded. All plants were reinfested with 10 newly hatched neonates taken directly from the IR22-SCPEC *N. lugens*. After these had developed to adults, mated adult females were placed on IR62 (2 females) and TN1 (2 females) for 24 hours and their honeydew production monitored (as indicated above) (**Figure 2**: Step 1; first generation). The remaining adults were placed on IR22 and allowed to lay eggs. The eggs were allowed to hatch and emerging nymphs were reared on IR22 plants until they became adults (**Figure 2**: Step 2). These adults were then collected (decendants of hoppers that had fed on the 30 original infested or non-infested Triveni plants) and their honeydew production on IR62 and TN1 plants was monitored (as indicated above) during 24h (**Figure 2**: Step 3; second generation). These adults were then dried at 60°C for three days and weighed.

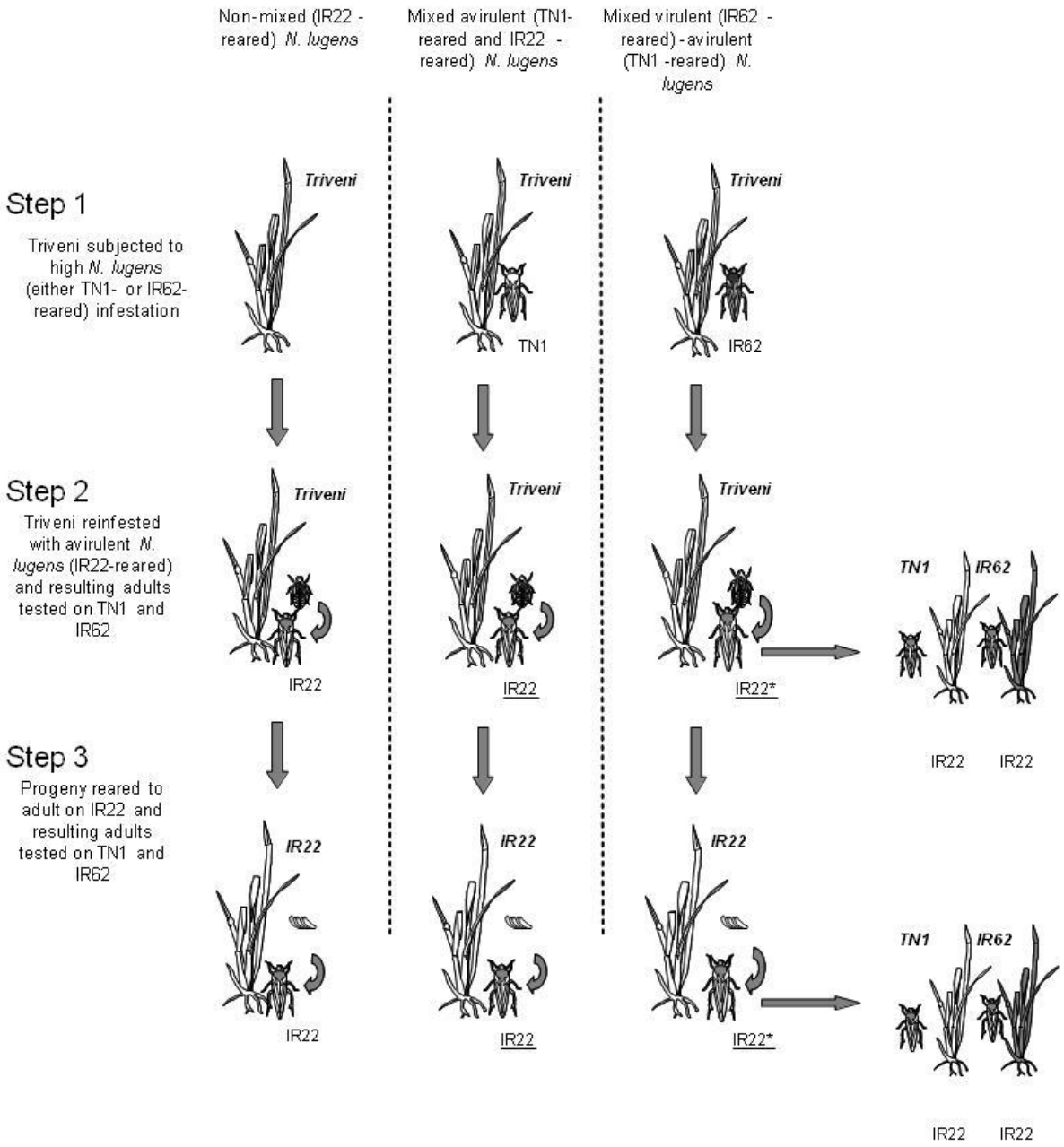

Figure 2. Procedure used in Experiment 2.

2.5 Data analysis

The biomass (mg dry weight) of nymphs reared on Triveni plants that were previously infested with planthoppers (from IR62-SCPEC or TN1-Laguna) were compared to nymphs reared on plants that were previously non-infested using a univariate General Linear Model (GLM) with above-ground plant biomass as a covariate. Honeydew production by adult planthoppers was examined for experiments 1 and 2 by univariate GLM. Xylem-derived honeydew as a proportion of the total amount of honeydew was used as an indicator of resistance with total honeydew as a covariate – this standardized for differences in insect size and in feeding activity. Data were ranked for experiment 2 and proportions were arcsine transformed. Because of large variability in the size of adult hoppers during experiment 2, we used the weight of planthoppers that fed on IR62 during the honeydew monitoring as a proportion of the weight of adult hoppers fed on TN1 during the same experiment. This gave a standardized metric of adaptation to IR62. Residuals were examined following all analyses (except where data had been ranked) and were found to be normal and homogeneous.

3.0 Results

3.1 Experiment 1: Responses by *N. lugens* to prior feeding by conspecifics on the same host plant

Nymphs reared on Triveni plants that were previously infested with *N. lugens* from the IR62 colony had the highest weight gains after 15 days, compared to nymphs reared on Triveni plants that were previously uninfested or previously infested with TN1-reared planthoppers. Nymph biomass was also highest, but to a lesser extent, when plants had been previously infested with TN1-reared *N. lugens* ($F_{2,24}$= 3.747, P = 0.041: **Figure 3A**). The covariate, above-ground plant biomass, also affected planthopper weights ($F_{1,24}$ = 12.012, P = 0.007) with weights of plants at post-feeding negatively related to planthopper biomass.

The amount of xylem excreted as honeydew by hoppers feeding on plants that were previously infested with IR62-adapted conspecifics was generally higher than for planthoppers feeding on plants that were not previously infested or were infested with TN1-reared planthoppers; however, this was not significant (proportion of xylem feeding: $F_{2,24}$ = 2.049, P = 0.155: **Figure 3B**). Honeydew quantity was negatively correlated with the proportion of xylem feeding (covariate: $F_{1,24}$ = 8.937, P = 0.007).

3.2 Experiment 2: Feeding response on IR62 and TN1 rice varieties by progeny of avirulent *N. lugens* from plants previously attacked by virulent conspecifics

During the first run of the experiment (run A), adults that developed from Triveni-reared neonates produced a higher amounts of xylem-derived honeydew when switched to IR62 compared to planthoppers that had been switched to feed on TN1. This occurred irrespective of feeding history (exposure to shared feeding sites and no exposure to shared feeding sites)(**Table 2**, experiment 2A). This pattern was maintained in the second generation (generation 1, adults developed from eggs laid on IR22). There were no apparent effects of feeding history on the relative weights of adults reared on IR62 or TN1: After feeding on IR62 for 24 hours, the adults attained 71% of the weight of those fed on TN1 in the first generation and ca 51% of the weight of those fed on TN1 in the second generation (**Table 2**, experiment 2A).

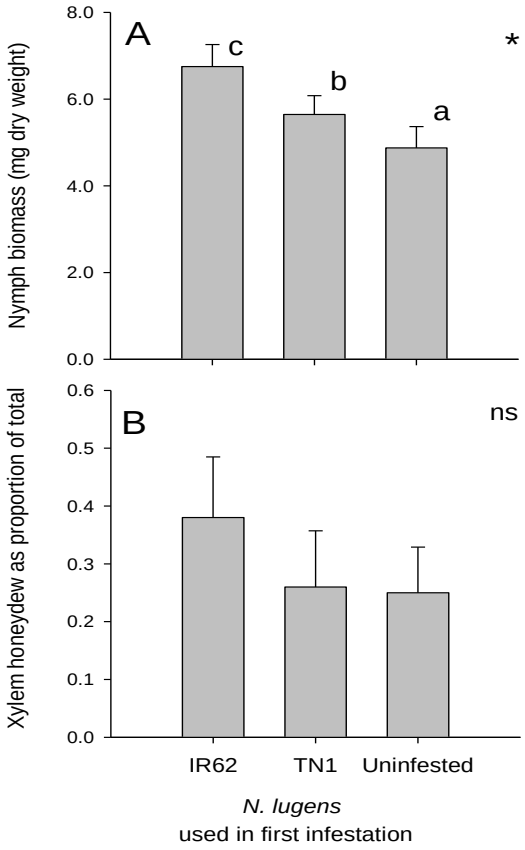

Figure 3. A) Weight of *Nilaparvata lugens* nymphs (after 15 days) reared on Triveni rice plants that had previously been infested with IR62-adapted or TN1-reared conspecific adults, or were not previously infested (uninfested) and B) Xylem-derived honeydew (collected over 24 hours) as a proportion of total honeydew excreted by adult female hoppers on the same plants. Test planthoppers had been continuously reared on IR22. N = 8 plants, * = $P < 0.05$, ns = $P > 0.05$. Standard errors are indicated.

Table 2. Results from two runs of experiment 2 (A and B): The progeny of *Nilaparvata lugens* that shared feeding plants with IR62-adapted (mixed with virulent) and TN1-reared (mixed with avirulent) conspecifics were tested on IR62 and TN1 after one and two generations without further exposure to mixed feeding sites. Numbers are means (SEM); N = 10 plants.

Feeding history	Same generation			Generation 2		
	Weight prop. (IR62/TN1)[5]	Xylem prop. IR62	Xylem prop. TN1	Weight prop. (IR62/TN1)[5]	Xylem prop. IR62	Xylem prop. TN1[4]
Experiment 2A						
Non-mixed	0.72 (0.04)	0.89 (0.07)	0.15 (0.04)	0.48 (0.02)	0.96 (0.04)	0.07 (0.02)
Mixed with avirulent	0.69 (0.06)	0.95 (0.04)	0.10 (0.03)	0.53 (0.04)	0.91 (0.05)	0.05 (0.01)
Mixed with virulent	0.75 (0.05)	1.00 (0.01)	0.11 (0.02)	0.54 (0.03)	0.98 (0.01)	0.06 (0.01)
F-Feeding history[1]	0.321ns	0.171ns		0.999ns	1.112ns	
F-test plant[2]		212.323***			213.043***	
F-interaction[3]		1.209ns			0.365ns	
Experiment 2B						
Non-mixed	0.82 (0.09)	0.25 (0.13)	0.10 (0.05)	0.80 (0.09)	0.49 (0.09)	0.21 (0.07)ab
Mixed with avirulent	0.90 (0.17)	0.34 (0.06)	0.15 (0.05)	0.90 (0.07)	0.76 (0.07)	0.35 (0.10)b
Mixed with virulent	0.95 (0.09)	0.13 (0.06)	0.07 (0.04)	1.08 (0.10)	0.54 (0.11)	0.15 (0.08)a
F-Feeding history[1]	0.260ns	5.071**		2.423ns	3.504*	
F-test plant[2]		3.082ns			23.624***	
F-interaction[3]		1.183ns			0.518ns	

1: Feeding history DF for weight proportions = 2, 27 (error); for xylem proportions = 2, 54 (error); ns = $P > 0.05$; * = $P \leq 0.05$; ** = $P \leq 0.01$

2: DF for test plants = 1, 54 (error); ns > 0.05; *** = $P \leq 0.001$

3: DF for interactions = 2, 54 (error); ns > 0.05

4: Letters indicate homogenous groups (Tukey test: $P \leq 0.05$)

5: Nymph weight on IR62 as a proportion of nymph weight on TN1 in the same experiments

During the second run of the experiment (run B), adults from Triveni-reared neonates (Same generation: Table 1) produced proportionally less xylem-derived honeydew when feeding on IR62 after they had shared feeding sites with IR62-virulent planthoppers. These planthoppers were also more virulent (producing less xylem) on TN1 compared with hoppers from the other two cohorts (Table 1). The same pattern was observed in the second generation of planthoppers (Table 1). After feeding on IR62 for 24 hours, the adults were 89% of the weight of those fed on TN1 in the first generation and 93% of the weight of those fed on TN1 in the second generation (Table 1); In both generations, planthoppers that had shared feeding sites with IR62-virulent populations attained the highest weights on IR62; however, the differences were not statistically significantly (Table 1). These results indicate that facilitation is potentially carried with the insect – such that feeding could be improved on novel (non attacked) plants and across generations of planthoppers. However, it is noteworthy, that the proportion of xylem-derived honeydew was significantly higher for all planthoppers on IR62 than on TN1 in the second generation, suggesting a decline over generations in the carry-over of virulence.

4.0 Discussion

A single rice plant may be repeatedly attacked by planthoppers during its lifetime. After first locating a suitable host, macropterous adult planthoppers generally initiate attacks by probing, feeding and laying eggs (Horgan, 2009). Where plants are attacked at an early rice-plant stage, up to three successive planthopper generations can develop. This can lead to plants with 1000s of individuals all interacting in a single space and feeding from the same phloem source. We suggested that such high-density feeding sites, as occur during planthopper outbreaks, could become focal points for the transfer of biological and genetic materials that influence rice-planthopper interactions. Our results indicate that the nature of

the rice plant may change depending on its recent history of attack. Triveni plants that had been previously attacked by virulent planthoppers (reared during >20 generations on IR62) were more favourable for nymph development than previously uninfested plants, or plants that had been infested by planthoppers with a different feeding history (in this case, over 30 generations on the susceptible variety TN1). Facilitation of non-virulent mites (*Tetranychus* spp.) by virulent mites on tomatoes (*Solanum lycopersicum*) has been noted in previous studies (Kant et al. 2004; Alba et al. 2015). Furthermore, different strains of mites differentially suppressed the plants defenses (Alba et al. 2015). However, in the present study – albeit not statistically significant - when adult planthoppers fed on Triveni rice with a history of previous attack, they tended to feed on xylem more than planthoppers infesting Triveni for the first time (i.e., no previous attack). Xylem feeding is normally considered an indicator of host plant resistance for *N. lugens*, which is a phloem feeder (Yoshihara et al. 1980; He et al. 2010), which may suggest that defenses in the previously infested plants were not significantly suppressed by the virulent planthopper colony.

Most phloem-feeding insects seem to be perceived by plants in a similar way to pathogens that induce the salicylic acid (SA)-signaling pathway causing the plants to produce pathogenesis-related proteins (PR) (Walling, 2000). Literature on rice resistance to planthoppers frequently suggests that rice plants will induce defensive responses to planthopper attacks (Karban and Chen, 2007). Specifically, planthoppers feeding on rice induce PR proteins (Kanno et al. 2005, Wang et al. 2005) and the salicylic acid pathway (Xu et al. 2003, Zhang et al. 2004). *Nilaparvata lugens* feeding also induces the expression of protease-inhibitor (PI) genes (Zhang et al. 2004; Wang et al. 2005), which affects protein digestion in the insect's midgut (Broadway et al. 1986; Jongsma and Bolter, 1997). Some of these studies have also attempted to link induced defenses to major resistance genes (Loka Reddy et al. 2004; Yuan et al. 2005; Hao et al. 2008; Wang et al. 2008a). Attack-elicitors

such as β-glucosidase present in the saliva of *N. lugens* have been linked to salicylic acid, hydrogen peroxide and ethylene production (Wang et al., 2008b). However, our study indicates that previous attack by planthoppers may facilitate feeding by conspecifics, and that induced facilitation is also a possibility. Facilitation of feeding by planthoppers that share a common host plant has been suggested for white-backed planthoppers (*Sogatella furcifera* [Hovarth]) and small brown planthoppers (*Laodelphax striatellus* [Fallen]) following brown planthopper attack (Cheng et al. 2001; Cao et al. 2013a,b) and spider mites (Kant et al. 2004; Alba et al. 2015). Furthermore, recent evidence from studies at IRRI suggests that facilitation may be the more common outcome of successive planthopper attacks (Horgan, unpublished) compared to induced defenses. To date, the mechanisms underlying facilitation have not been elucidated.

Of particular interest in this study is the observed difference in outcome of the rice-planthopper interactions depending on whether the host plant was previously infested by virulent or avirulent planthoppers. This suggests that plant responses are specific to the population of planthoppers that initially attack the plant. Ji et al. (2013) found differentially expressed genes associated with the saliva of two populations of *N. lugens* [reared on either TN1 (susceptible) or Mudgo (carrying the *Bph1* gene)], suggesting that the quality of the planthoppers may affect the plant's response in a manner similar to the facilitation of non-virulent mites (*Tetranychus* spp.) by virulent mites on tomatoes (*Solanum lycopersicum*) (Kant et al. 2004; Alba et al. 2015).

The present study employed bioassays to evaluate planthopper virulence. We suggest that if no effect can be seen in the insect responses to a plant's history of attack, then there is little need to examine the hypothesis using molecular methods. However, bioassay results must be interpreted with caution: poor feeding by adult planthoppers on pre-infested Triveni

rice might be time dependent. We observed honeydew production during 24h, but planthoppers might improve feeding over longer time periods. Nymph biomass is a better test of virulence and gave the clearest results in this study. We recommend that future studies of this kind would better monitor feeding responses using more sensitive devices such as Electrical Penetration Graphs (Seo et al. 2009; Cao et al. 2013a,b), and continue observations for longer. Molecular or microbial determination of the potential mechanisms underlying horizontal (planthopper to planthopper) facilitation of virulence will be useful when bioassays can be optimized.

Several researchers have previously reported the presence of large numbers of bacteria-like organisms (BLOs) in the salivary sheaths of *N. lugens* as well as at planthopper feeding sites in rice plants (Wang et al. 2008; Tang et al. 2010). Such orally-secreted microbial symbionts might trigger the plant into activating the SA pathway that channels defenses toward bacteria and relaxes potential defenses against the insect as the plants are unable to effectively activate the jasmonic acid defenses due to negative cross talk with the SA defenses (Chung et al. 2013). Tang et al. (2010) have indicated that planthoppers reared on different rice varieties had distinct bacterial communities, giving some credence to a role for bacteria in determining virulence. Together, these observations support the VAH that suggests that planthoppers may gain virulence against resistant rice varieties by acquiring virulence factors indirectly through host plants on which virulent planthoppers contemporaneously feed or had previously fed. Such virulence factors might include symbiotic microbes; however, further evidence is required to prove this.

Our second experiment indicated virulence was horizontally acquired in our system after intense feeding by virulent planthoppers on the tolerant variety: planthoppers, that had shared feeding sites with virulent conspecifics were facilitated during feeding on the shared

host plant (experiment 1) and demonstrated an increased virulence when transferred to new plants (IR62 or TN1: experiment 2). Furthermore, the feeding responses by the planthoppers were similar among same-generation and progeny planthoppers in our second experiment (suggesting that virulence factors may be vertically transmitted, possibly by contamination of eggs, or plant surfaces). Therefore, our results, where planthoppers apparently acquired virulence by picking-up unknown virulence factors (OS or BLOs) during feeding at high-planthopper density feeding sites with mixed virulent-avirulent planthopper populations supports the VAH. However, we suggest that further detailed studies of the mechanisms underlying virulence acquisition are required.

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CHAPTER 6

Comparative performance of virulence-adapted and non-adapted *Nilaparvata lugens* on different rice varieties

Jedeliza B. Ferrater, Fay Haverkort, Reyuel Quintana, Maria Liberty P. Almazan, Peter W. de Jong, Finbarr G. Horgan

Abstract

This study examines the levels of resistance against Philippine populations of the brown planthopper, *Nilaparvata lugens* (Stål), using a large collection of rice differentials, representing 12 resistance genes and 2 major Quantitative Trait Loci (QTLs). We found that Philippine planthoppers were able to feed and lay eggs on all the varieties we tested. However, varieties with the the *Bph3* or *bph4* genes and the *Bph9* gene ranked highest in resistance against *N. lugens*. We further addressed two hypotheses: First, that planthoppers adapted to resistant varieties would have increased fitness on similar resistant varieties, i.e., varieties with the same or closely related resistance genes. Second, that planthoppers adapted to resistant varieties would experience fitness costs that reduce their ability to feed on unrelated or dissimilar varieties. We examined the relative fitness of planthoppers from colonies that had been selected for more than 20 generations on resistant and susceptible varieties, when these were switched to feed and oviposit on a range of new varieties, representing a range of resistance genes. We observed that planthoppers that had adapted to resistant varieties (with *Bph3*) had an increased fitness on varieties with the same or closely related resistance genes; however, evidence for fitness costs was generally weak. The results of this study emphasize the need for effective deployment of resistant rice varieties to avoid rapid adaptation by planthoppers.

1.0 Introduction

The development of herbivore-resistant crops through conventional breeding is regarded as a potentially efficient means to reduce crop losses among resource poor farmers (Panda and Khush, 1995; Savary et al. 2012). National programs can make the seed of resistant crop varieties freely available to farmers, allowing them to sustainably reduce costs related to pest management (Widawsky et al. 1998; Savary et al. 2012). Several resistant crop varieties are available to farmers including potato (*Solanum* spp.) with resistance against tuber moths (Lepidoptera: Gelechiidae) and aphids (Hemiptera: Aphididae)(Pelletier et al. 2011), wheat (*Triticum* spp.) with resistance against Hessian fly (*Mayetiola destructor* [Say]: Cambron et al. 2010) and aphids (Porter et al. 2000), lettuce with resistance to aphids (Eenink et al. 1982) and legumes with resistance against a range of insects and mites (Edwards and Singh, 2006). Host plant resistance against rice pests has received considerable and constant attention over the last several decades (Fujita et al. 2013; Horgan and Crisol, 2013). The development of insect-resistant, high-yielding rice varieties is important given that rice is consumed by more people than any other crop, particularly in the developing nations of Asia, Africa, Latin America and the Caribbean, and because a large proportion of the World's estimated 100 million rice farmers are considered resource poor (IRRI, 2008). Rice varieties with resistance against gall midge (*Orseolia* spp. - Diptera: Cecidomyiidae), planthoppers (Hemiptera: Delphacidae), leafhoppers (Hemiptera: Cinindelidae) and caterpillars (Lepidoptera) have all been recognized or developed in recent years (Panda and Khush, 1995; Fujita et al. 2013; Horgan and Crisol, 2013).

The brown planthopper (*Nilaparvata lugens* [Stål]) is considered among the most damaging pests of rice in Tropical and Subtropical Asia (Bottrell and Schoenly, 2012). Prior to the Green Revolution of the 1960s, the brown planthopper was an occasional pest in North East Asia (China, Japan and Korea) during rare weather events that caused windblown

migrating planthoppers to converge to restricted areas of rice (Sogawa, 1982; Bottrell and Schoenly, 2012). In Tropical Asia, modern high-yielding rice varieties that were susceptible to the planthopper, high fertilizer use, and resurgence-causing pesticides have been responsible for sustained outbreaks that often result in considerable losses to rice productivity (Kenmore et al. 1987; Heong et al. 1994; Bottrell and Schoenly, 2012). For example, it is estimated that during 2009, over one million hectares of rice in Central Thailand was destroyed by *N. lugens*, prompting the Thai government to ban the use of the resurgence-causing pesticides Abamectin and Cypermetrin on rice (IRRI Media Release, 2011). In 2011, an estimated 200 thousand hectares of rice was destroyed in Central Java as a result of planthoppers and planthopper-transmitted viruses (Horgan, unpublished data).

Over the last 50 years, a range of modern, high-yielding rice varieties have been developed with resistance against planthoppers: The first of these – IR26, with the *Bph1* resistance gene, was released by the International Rice Research Institute in 1973; however, within a few years after its release, planthopper populations had adapted to feed on varieties with *Bph1*-derived resistance. This prompted the release of IR36 with the *bph2* resistance gene, against which planthoppers also rapidly adapted (Horgan, 2012). Varieties with the *Bph3* resistance gene were subsequently released and appear largely durable albeit with generally low farmer adoption (Peñalver Cruz et al. 2011). Recently, a series of studies have demonstrated widespread adaptation by planthoppers to formerly resistant varieties that possess a range of resistance genes (Myint et al. 2009a,b; Fujita et al. 2013; Ferrater et al. 2015 [Chapter 3]). Some of these genes (i.e., *BPH25* and *BPH26*: Srinivasan et al. 2015) have never been widely deployed in farmers' fields suggesting that virulence adaptation against one resistant variety may reduce the effectiveness of multiple resistance genes even without their widespread deployment.

In this study, we examine levels of resistance against Philippines *N. lugens*, across a range of varieties with known resistance genes. Currently some 80 'hopper' resistance genes have been identified of which about 34 demonstrate moderate to high resistance against *N. lugens* (Fujita et al. 2013). We acquired a large collection of varieties, representing at least 12 resistance genes and 2 major Quantitative Trait Loci (QTLs). We also included two suggested tolerant varieties Triveni and Utri Rajapan (Ho et al. 1982; Panda and Heinrichs, 1983), and the susceptible controls IR22 and Taichung Native 1 (TN1: De Datta, 1981). Furthermore, we examined the effects of adaptation in planthoppers on the wider effectiveness of resistant rice plants. We addressed two potential hypotheses: Firstly, that planthoppers adapted to resistant varieties would have increased fitness on similar resistant varieties, i.e., varieties with the same or closely positioned resistance genes. Secondly, that planthoppers adapted to resistant varieties would experience fitness costs that reduce their ability to feed on unrelated or dissimilar varieties. These hypotheses are not mutually exclusive. For the purpose of this study, we assume that the presence of particular major resistance genes largely determines similarity or difference among rice varieties for a monophagous species like *N. lugens*; however, we recognize that the genetic distance between varieties, particularly varieties without any major resistance genes, also plays a role in the feeding preferences and resulting fitness of planthoppers across varieties (Horgan, 2012). We tested the two hypotheses by examining the relative fitness of planthoppers, from colonies that had been selected for several generations on resistant varieties, when these were allowed to feed or oviposit on a range of varieties across a gradient of resistance. We discuss our results in the light of effective deployment of resistant rice varieties.

2.0 Materials and Methods

2.1 Plant materials

Several wild rice species (e.g., *Oryza australiensis* Domin) and traditional rice varieties are resistant to the brown planthopper (Fujita et al. 2013). Many of these have been used as donor parents to develop modern resistant varieties using traditional breeding methods. Furthermore, Marker Aided Selection (MAS) has recently been used to develop Near-Isogenic Lines (NILs) containing known resistance gene loci. We selected a range of rice varieties (including traditional varieties, modern varieties with wild or traditional resistance donors, and resistant NILs) to examine aspects of rice resistance and planthopper adaptation. Table 1 lists the varieties that were used in the experiments and gives details of their origins and pedigrees, as well as their putative resistance genes. These were chosen to represent as many resistance genes as possible using plants with good germination and survival under tropical conditions. Many of the varieties are standard varieties commonly employed as resistance donors in rice breeding programs. All seeds were acquired from the International Rice Research Institute (IRRI). Traditional varieties were acquired through the International Network for Genetic Evaluation for Rice (INGER). The IR varieties were acquired through the Plant Breeding, Genetics and Biotechnology (PBGB) Division of IRRI. One breeding line – IR65482-7-216-1-2 – was included as two batches (including a batch received directly from KK Jena [IRRI]) because of apparent discrepancies between observed levels of resistance with the INGER supplied line indicative of genetic erosion of resistance. For the experiments reported here, the seed was multiplied in a screenhouse facility at IRRI during November and December 2010 and stored in a cold room (4^{o}C) until use in the experiments. Prior to the experiments, the seeds were incubated for 72 hours at 60^{o}C to break dormancy. After incubation, seeds were soaked in water for 24 hours and then transferred to Petri dishes lined with moistened absorptive paper for 72 hours. The Petri dishes were

incubated in a dark room at ambient temperature (28^{o}C). Germinated seedlings were transplanted to soil in pots (7×11cm; H×D [diameter]) for nymphal survival and egg-laying bioassays (see below). Larger pots (22×24cm; H×D) were used for the population build-up bioassays (see below). All experiments were conducted in a greenhouse at IRRI. Temperatures in the greenhouse ranged between 26 and 45^{o}C over the course of the study, with natural daylight regimes (light from ca. 6am to 6pm throughout the year).

2.2 Insect Populations

We used planthoppers from several laboratory colonies maintained at IRRI. The *N. lugens* populations were originally collected from Philippine rice paddies between September and November of 2009. The insects collected consisted of between 50-500 individuals from each of three geographical locations: Mindoro [13.00° N, 121.08° E], Iloilo [10.72° N, 122.57° E], and South Cotabato - Mindanao [6.17° N, 125.00° E]). After five generations of building up populations, 200 gravid females of each of the three populations were collected and 100 each placed on four rice varieties (IR65482-4-136-2-2, IR62, IR22, and PTB33) for selection (a total of 12 colonies). The breeding line IR65482-4-136-2-2 contains the resistance gene *Bph10* derived from the wild rice *O. australiensis* (Fujita et al. 2013). PTB33 is a traditional Indian variety considered among the most resistant rice varieties to Philippine populations of *N. lugens.* It has been used extensively in breeding programs aimed at increasing rice resistance to biotic stresses. The genetics of resistance in PTB33 is still not fully understood, but is likely governed by a number of major resistance genes (including *bph2* and *Bph3*) (Angeles et al. 1986) and several minor genes (Santhanalakshmi et al. 2010). IR62 is a modern rice variety released by IRRI in 1984. The variety is highly resistant to *N. lugens* from Laguna, Philippines (used in IRRI's resistance breeding program). Its resistance has been attributed to the *Bph3* gene that was likely obtained from PTB33 (Peñalver Cruz et al. 2011). IR22 is thought to contain no major resistance genes (Brar et al. 2009). The 12

experimental colonies were continuously maintained on the differential varieties during 20 generations of selection (approximately 24 months) during which time they were held in an insectary with a 12h:12h L:D regime and a constant temperature of ca. 26^{o}C. Further details of the selection procedure can be found in Ferrater et al. (2015; Chapter 3). To avoid potential problems associated with inbreeding after 20 generations of rearing on the different varieties, those colonies reared on the same host variety but with different geographical origins were introgressed as a single colony on that same variety. This produced four colonies (henceforth the South-Central Philippines Experimental Colonies, SCPEC), one each reared on IR65482-4-136-2-2, IR62, IR22, or PTB33. To combine colonies, newly emerged adult males and females (100-200 from each colony) were collected after generation 20 and placed on their designated host plants (according to the plant on which they were selected) in single large aluminum wire mesh cages of 91.5 × 56.5 × 56.5 cm (H×L×W) in a greenhouse at IRRI. A further colony, collected at Laguna (14.17° N, 121.33° E) that had been maintained in large cages for ca. 30 generations on TN1 was used as a further test colony (henceforth the 'TN1-Laguna' colony). This colony did not experience population decline during rearing or maintenance and was therefore not introgressed with other similar colonies.

2.3 Baseline responses by *N. lugens* to resistant rice varieties

We examined resistance across the 24 varieties in a series of bioassays. Planthoppers from two colonies (IR22-SCPEC and TN1-Laguna) that had not been exposed to resistant varieties during 20-30 generations were used for the tests. The bioassays were conducted during 2012 and 2013 as two temporal replicates for each planthopper colony. Furthermore, each bioassay was replicated five times in a completely randomized design during each temporal replicate. The following bioassays were conducted to evaluate planthopper responses:

Nymph survival: To determine the performance of nymphs on each variety, 10 newly emerged nymphs were placed together on 15-day-old plants. Plants were produced from pre-germinated seedlings in clay pots (7×11cm; H×D) each enclosed in a cylindrical mylar cage (61×10.5cm; H×D) with a mesh side window and top for ventilation (**Figure 1A**). After 15 days, the survivors were collected and oven-dried at 60ºC for 3 days. The above-ground plant parts (shoots) were cut, placed in paper envelopes and oven-dried at 60ºC for at least 7 days before being weighed to estimate plant biomass.

Oviposition performance: The number of eggs laid on each variety was determined by confining two gravid female *N. lugens* on 15-day-old plants for 3 days. Plants were produced from seedlings in clay pots (7×11 cm; H×D) each enclosed in a cylindrical mylar cage (61×10.5cm; H×D) with a mesh side window and top for ventilation. After 3 days, the insects were removed and the plants were collected and frozen at -20^{o}C. These plants were later dissected and the number of eggs laid on each plant was counted under a stereomicroscope (10× magnification).

Biomass build-up: Two gravid female *N. lugens* were confined on 30-day-old plants in pots (22×24 cm; H×D). The rice plants (and insects) were enclosed in organza cages (150×22cm; H×D). The organza cloth was fitted around a cylindrical mylar base cage (30×22cm; H×D) stably embedded in the soil inside the pot and supported by bamboo stakes and aluminium wire rings. The top loose end of the cloth was tied to confine the insects (**Figure 1B)**. The females were left to lay eggs and the emerging nymphs were allowed to develop for 30 days. Planthoppers present in the cages after 30 days were collected using a mechanical aspirator and oven-dried during 3 days at 60^{o}C before being weighed. After collecting the insects, the rice shoots were collected, cleaned of soil, placed in paper bags and oven-dried at 60ºC for 7 days before being weighed.

The nymph survival and oviposition performance bioassays were carried out in a greenhouse at temperatures ranging from 25-45^{o}C. The biomass build-up bioassay was conducted in a screenhouse facility with temperatures of 25-37^{o}C. All bioassays were replicated five times, for each of the two colonies and with two temporal replicates.

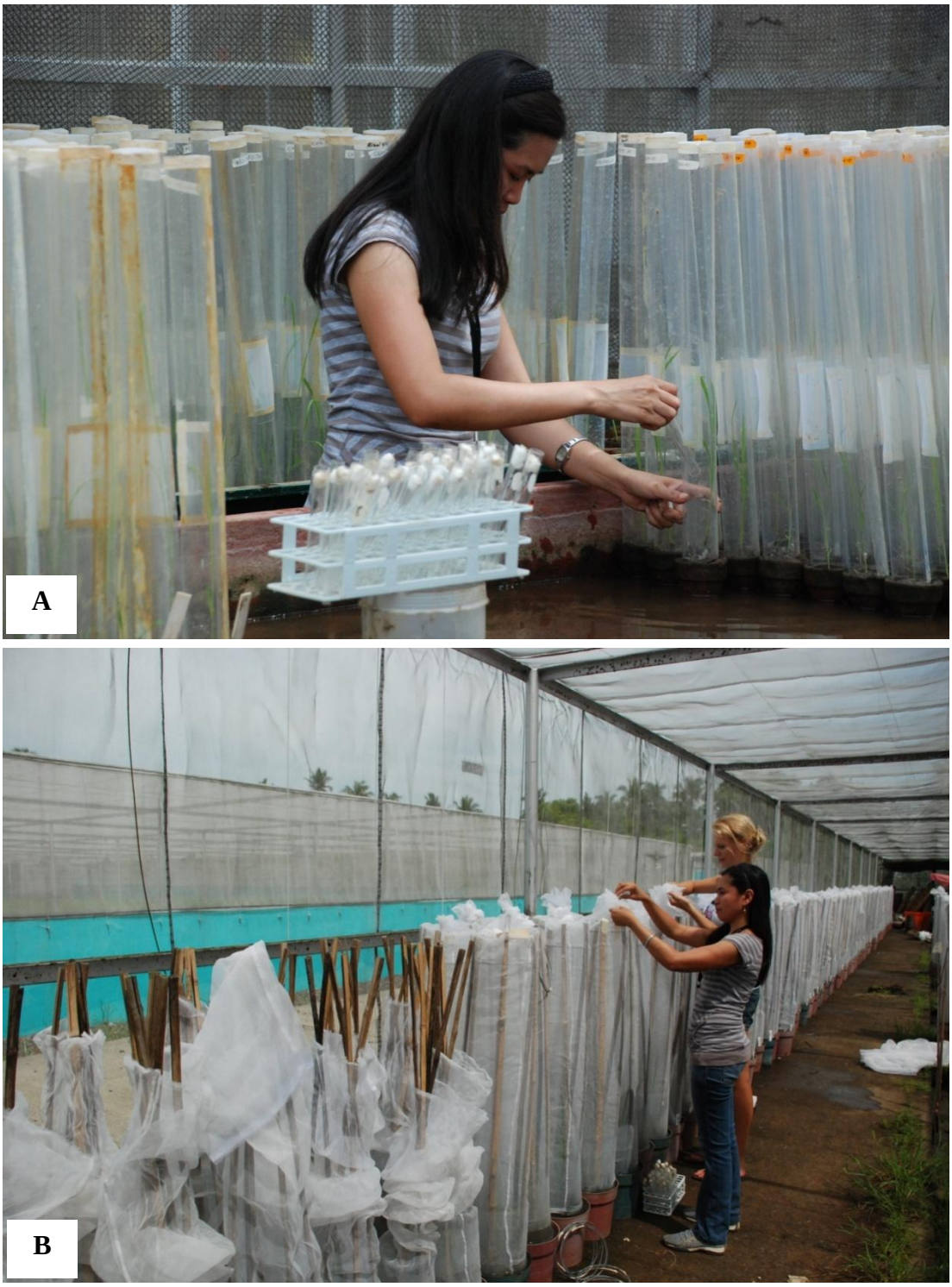

Figure 1. Performance bioassays for *Nilaparvata lugens* on different rice varieties. A) set-up for nymphal survival and oviposition performance B) set-up for biomass build-up.

2.4 Performance of PTB33-adapted and non-adapted *N. lugens* on different rice varieties

We compared the performance of planthoppers that had been selected during several generations on the highly resistant rice variety PTB33 (*bph2*, *Bph3*, and other genes), with performance by planthoppers that had been maintained on the highly susceptible variety IR22 during several generations. Planthoppers from the two colonies (PTB33-SCPEC and IR22-SCPEC) were examined for performance on the varieties (**Table 1**) using the parameters indicated above (nymph survival, oviposition performance, and biomass build-up). The bioassays were each replicated five times in a completely randomized design. Nymph survival and oviposition bioassays were conducted in a greenhouse, whereas the biomass build-up experiment was conducted in a screenhouse as described above. The entire experiment had two temporal replications.

2.5 Performance of IR62-adapted, IR65482-4-136-2-2-adapted, and non-adapted *N. lugens* on different rice varieties

Planthoppers selected on IR62 (*Bph3*) and IR65482-4-136-2-2 (*Bph10*) were examined for their performance on a range of varieties (Table 3). The colonies (IR62-SCPEC and IR65482-4-136-2-2-SCPEC) were also compared with the TN1-Laguna colony that had been reared for 30+ generations on the susceptible host TN1. The performance of planthoppers from each colony was examined using the three parameters (nymph survival, oviposition performance, and biomass build-up) indicated above. The rice variety TN1 was included in the bioassays as a susceptible control. The bioassays were each replicated five times in a completely randomized design. Nymph survival and oviposition bioassays were conducted in a greenhouse, whereas the biomass build-up bioassays were conducted in a screenhouse as described above.

2.6 Data analyses

We examined the response variables (eggs laid, nymph survival and growth [nymph biomass], and biomass build-up [population biomass]) for *N. lugens* on the range of rice varieties using univariate General Linear Models (GLM). Data was ranked within colonies and temporal repetitions to standardize for variable conditions during the greenhouse and screenhouse experiments. The independent variables initially included in the models were test colony (IR22-SCPEC and TN1-Laguna), repeat (2012, 2013), and exposed variety (the range of different varieties). Plant weight was included as a covariate. We backward eliminated non-significant factors. Tukey tests were used to determine homogeneous groups. Average resistance rank was calculated as the average ranking (where 1 is most resistant and 24 the least resistant) from each of the three fitness parameters. We examined relations between the three fitness parameters using Pearson correlations. Residuals were examined after analyses and found to be normal and homogeneous.

Performance parameters of the PTB33-SCPEC planthoppers to the varieties were compared against responses by planthoppers from IR22-SCPEC using univariate GLM. Because we were interested in the overall trends across varieties for each colony, we ranked the response data for each variable within repeats and test colonies (PTB33-SCPEC or IR22-SCPEC). The GLM model examined the effects of repeat, colony and variety on ranked performance. Significant colony × differential variety interactions would indicate changes in the relative performance by the adapted colony (PTB33-SCPEC) on the different varieties compared to the non-adapted colony (IR22-SCPEC). Where such changes were apparent, we used Mann-Whitney U tests to examine the original data for significant differences in performance by planthoppers from the two colonies.

Performance parameters of planthoppers from the IR62-SCPEC, IR65482-4-136-2-2-SCPEC and TN1-Laguna colonies to 17 different varieties were examined using univariate

GLM. The parameters 'biomass build-up' and 'nymph biomass' were standardized for variability in plant biomass for this experiment (see **Table 3**) because of extensive damage caused by IR62-SCPEC planthoppers on some varieties. Data residuals were examined after analyses and found to be normal and homogeneous. Tukey tests were used to determine homogeneous groups.

3.0 Results

3.1 Baseline performance of *N. lugens* on the different rice varieties

Planthopper egg laying, nymph biomass and population development (biomass) showed generally similar responses across the 24 varieties (**Table 1**, **Table 2**).

Varieties with the the *Bph3* or *bph4* genes (Rathu Heenati, PTB33, Babawee, and IR62) and the *Bph9* gene (Balamawee) ranked highest in resistance. Compared to the standard susceptible control TN1, planthoppers on Rathu Heenati had significantly lower nymph and population biomass, Balamawee had lower egg numbers and nymph biomass, PTB33 had lower nymph biomass and IR62 had lower egg numbers (**Table 1**). Compared to the highly susceptible variety IR22, planthoppers on Rathu Heenati had significantly lower egg numbers, nymph and population biomass, planthoppers on Balamawee and PTB33 had lower egg numbers and population biomass and planthoppers on IR62 laid fewer eggs (**Table 1**). Egg laying by hoppers on Swarnalata (*Bph6* gene) was also lower than significantly on IR22. The three performance parameters were highly correlated across the range of varieties (**Table 2**).

3.2 Performance by PTB33-adapted and non-adapted *N. lugens* on different rice varieties

The rank order of resistance to *N. lugens* across varieties was generally similar for PTB33-SCPEC and IR22-SCPEC planthoppers (**Figure 2**). As with the previous experiment, varieties with the *Bph3* gene generally ranked highest in resistance; however, only Rathu

Heenati (nymph biomass) was significantly more resistant than IR22 and TN1 ($F_{22,404}$ = 6.621, P < 0.001), Balamawee remained more resistant than TN1 for egg laying ($F_{23,411}$ = 4.202, P < 0.001) and PTB33 remained more resistant for population biomass ($F_{18,163}$ = 2.427, P = 0.002) for both colonies. Planthopper colony had no effect on the relative nymph-biomass ($F_{1,404}$ = 2.037, P = 0.154), egg laying ($F_{1,411}$ = 0.031, P = 0.0861), or population biomass ($F_{1,163}$ = 0.024, P = 0.878) parameters. However, for all performance parameters the relative ranking on many of the most resistant varieties increased whereas ranking of the susceptible varieties tended to decrease when planthoppers were adapted to PTB33 – this produced a significant colony by differential variety interaction for nymph biomass ($F_{22,403}$ = 1.612, P = 0.041) mainly because of the lower average relative ranking of nymph biomass on Balamawee and Rathu Heenati (**Figure 2A**). There were no significant interactions for the other fitness parameters.

Table 1. Modern varieties, and rice breeding lines with known anti-herbivore resistance genes, known susceptibility or suggested tolerance to *Nilaparvata lugens*. Varieties are listed in order of their average resistance rank, as determined in this study. Numbers in bold font indicate significant reduction in fitness compared to the standard susceptible varieties TN1 or IR22.

Varieties	Origin and development	*N. lugens* resistance gene(s)	Nymph biomass (mg)††	Number of eggs per plant††	Planthopper biomass (mg) after 30 days††	Average resistance rank
Rathu Heenati[1]	Traditional variety, Sri Lanka	*Bph3*	**0.99 (0.21)^{ab}**	**37.60 (5.80)^{ab}**	**11.95 (4.70)^{a}**	2.00
Balamawee[2]	Traditional variety, Sri Lanka	*Bph9*	**0.81 (0.21)^{a}**	**36.85 (9.57)^{a}**	**16.72 (9.82)^{ab}**	2.00
PTB33[3]	Traditional variety, India	*bph2, Bph3, Bph17*	**1.15 (0.19)^{abc}**	**39.05 (11.75)^{abc}**	**13.37 (6.71)^{ab}**	3.00
Babawee[4]	Traditional variety, Sri Lanka	*bph4*	1.31 (0.28)^{abcde}	47.60 (11.28) ^{abcd}	18.83 (6.78)^{abc}	5.00
IR62[1]	Released by IRRI in 1984 (Philippines)	*Bph3*	1.50 (0.27)^{abcdef}	**32.92 (8.82)^{a}**	44.30 (20.09)^{abc}	5.67
IR65482-4-136-2-2[5]	IRRI breeder's line developed through introgression with *Oryza australiensis* Domin	*Bph10*	1.22 (0.20)^{abcd}	57.10 (13.16)^{abcd}	36.04 (18.03)^{abc}	6.67
IR66[1]	Released by IRRI in 1987 (Philippines)	*bph4*	1.67 (0.21)^{abcdef}	53.15 (16.33)^{abcd}	52.15 (18.49)^{abc}	9.67
Triveni[6]	Traditional variety, India	None (tolerance)	1.44 (0.21)^{abcdef}	59.50 (21.00)^{abcd}	59.81 (18.48)^{abc}	10.00
T65-NIL-*BPH25*[7]	Near-isogenic line developed at Kyushu University, Japan, using the resistance donor ADR52 (India) with the recurrent parent T65	*BPH25*	1.96 (0.22)^{cdef}	49.00 (18.39)^{abcd}	42.51 (27.32)^{abc}	10.67
IR65482-7-216-1-2†[8]	IRRI breeder's line developed through introgression with *Oryza australiensis* Domin	*Bph18*	1.55 (0.19)^{abcdef}	91.87(17.86)^{cd}	25.16 (6.38)^{abc}	12.00
Swarnalata[2]	Traditional variety, Bangladesh	*Bph6*	1.78 (0.26)^{bcdef}	**42.95 (11.49)^{abc}**	75.25 (20.09)^{bc}	12.00
IR40[9]	Released by IRRI in 1977 (Philippines)	*bph2*	1.61 (0.19)^{abcdef}	65.62 (18.51)^{abcd}	92.14 (39.18)^{abc}	13.33

ASD7[10]	Traditional variety, India	*bph2*	1.91(0.30)^{bcdef}	67.00 (21.19)^{abcd}	62.80 (21.92)^{abc}	14.33
Chinsaba[2]	Traditional variety, Myanmar	*bph8*	2.01 (0.24)^{cdef}	81.10 (22.19)^{abcd}	32.77 (16.50)^{abc}	14.67
Mudgo[11]	Traditional variety, India	*Bph1*	1.76 (0.31)^{bcdef}	89.45 (23.19)^{abcd}	56.22 (18.34)^{abc}	15.67
T65-NIL-*BPH26*[7]	Near-isogenic line developed at Kyushu University, Japan, using the resistance donor ADR52 (India) with the recurrent parent T65	*BPH26*	2.00 (0.22)^{def}	83.27 (0.00)^{abcd}	47.91 (26.23)^{abc}	16.00
IR65482-7-216-1-2[8]	IRRI breeder's line developed through introgression with *Oryza australiensis* Domin	*Bph18*	2.46 (0.15)^{f}	66.85 (0.00)^{abcd}	53.78 (31.87)^{abc}	16.00
IR22[12]	Released by IRRI in 1978 (Philippines)	None	1.67(0.37)^{abcdef}	76.80 (11.17)^{d}	124.37 (39.66)^{c}	17.00
Utri Rajapan[13n]	Traditional variety, Indonesia	None (tolerance)	1.95 (0.25)^{bcdef}	67.40 (19.47)^{abcd}	106.75 (31.47)^{abc}	17.33
IR24[12]	Released by IRRI in 1978 (Philippines)	*Bph1*	1.76 (0.24)^{abcdef}	87.96 (14.80)^{cd}	89.75 (22.78)^{abc}	17.67
Yagyaw[14]	Traditional variety, Vietnam	*Qbph3, Qbph9*	2.03(0.24)^{cdef}	75.06 (15.86)^{abcd}	87.16 (30.65)^{bc}	18.00
IR64[9]	Released by IRRI in 1985 (Philippines)	*Bph1, ++*	2.24 (0.24)^{f}	79.10 (19.86)^{cd}	74.26 (23.82)^{abc}	18.67
IR74[1]	Released by IRRI in 1988 (Philippines)	*Bph3*	2.25 (0.13)^{ef}	72.6 (10.80)^{cd}		19.00
Taichung Native 1 (TN1)[1]	Released in Taiwan in 1960	None	2.07 (0.33)^{def}	102.5 (27.78)^{bcd}	98.73 (43.12)^{bc}	22.00
F-value			5.960***	4.713***	3.412***	
Df (numerator)			24	23	22	
Df (denominator)			377	409	252	

Source references - 1: Peñalver Cruz et al. 2011; 2: Nemoto et al. 1989; 3: Santhanalakshmi et al. 2010 ; 4 : Lakshminarayana and Khush 1977; 5: Ishii et al. 1994 ; 6 : Ho et al. 1982 ; 7 : Fujita et al. 2013; 8: Jena et al. 2006 ; 9 : Khush and Virk 2005; 10: Athwal et al. 1971; 11: Sidhu et al. 2005; 12: Schiller et al. 2006; 13: Velusamy et al. 1986; 14: Liu et al. 2009; †: Two batches of IR65482-7-216-1-2 were used in these experiments – the seed used here was supplied by KK Jena (IRRI); ; ††: Lowercase letters indicate homogeneous groups according to Tukey multiple comparison tests; *** = $P < 0.001$

Table 2. Pearson correlation coefficients (above diagonal) and corresponding P-values (below diagonal) for three fitness parameters on a range of rice varieties

	Egg laying[1]	Nymph (biomass)[1]	Population (biomass)[1,2]
Egg laying	-	0.661	0.717
Nymph (biomass)	< 0.001	-	0.577
Population (biomass[2])	0.004	< 0.001	-

1: Data log+1-transformed

2: N = 23, otherwise N = 24

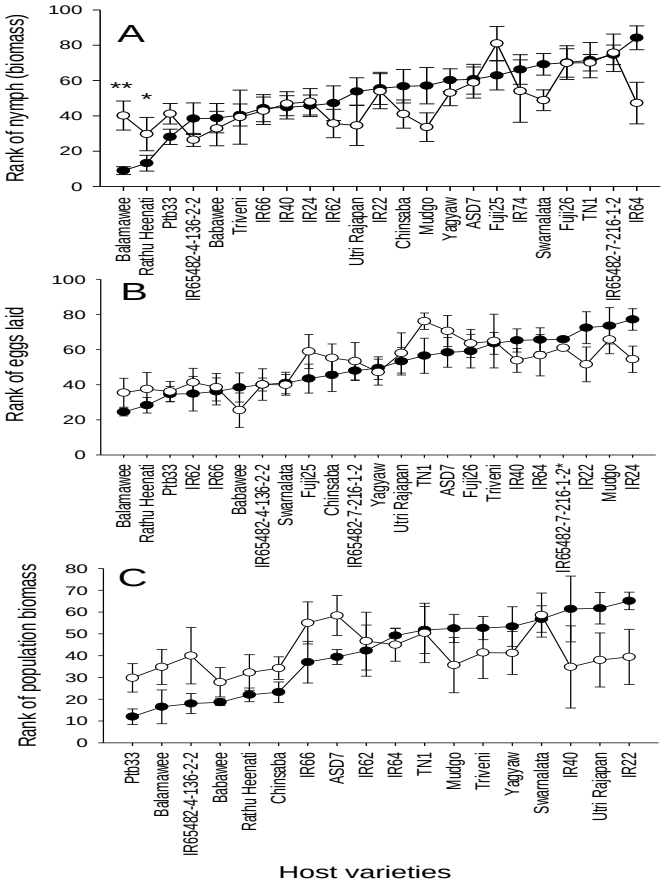

Figure 2. The rank order of resistance to *N. lugens* for PTB33-SCPEC (open circles) and IR22-SCPEC (shaded circles). Relative resistance is indicated as A) rank nymph biomass B) ranks eggs laid C) rank population biomass * = P < 0.05, ** = P < 0.01 (univariate GLM).

3.3 Resistance of rice varieties to *N. lugens* from IR62-SCPEC and IR65482-4-136-2-2-SCPEC colonies

There was no difference in egg laying by planthoppers from the TN1-Laguna colony on the different varieties when compared to planthoppers from the IR62-SCPEC and IR65482-4-136-2-2-SCPEC colonies. However, overall egg-laying by planthoppers from IR62-SCPEC was significantly lower than by hoppers from IR65482-2-136-2-2-SCPEC (**Table 3**). Nymphs from the IR62-SCPEC caused greater losses in plant biomass per dry weight of insect when compared to hoppers from the other two colonies. Unlike IR62-SCPEC planthoppers, the IR65482-4-136-2-2-SCPEC and TN1-Laguna planthoppers caused no reduction in plant weight on PTB33, Babawee or Rathu Heenati. Plant biomass reduction due to the IR62-SCPEC planthoppers was also more severe for most of the other rice varieties when compared to damage from the other two colonies (**Table 3**). These differences produced a significant colony by variety interaction for plant weight loss ($P < 0.05$). The rank order of resistance (which may include aspects of plant tolerance) to damage by nymphs from the IR62-SCPEC markedly decreased in Rathu Heenati (TN1 = rank 3, IR65482-2-136-2-2 = rank 2, IR62 = rank 11), but increased in ASD7 (TN1 = rank 12.5, IR65482-2-136-2-2 = rank 16, IR62 = rank 4) compared to variety resistance rankings for the other two planthopper colonies. Colony had no significant effect on planthopper biomass build-up on the different rice varieties. Across varieties and parameters, PTB33, Babawee, Rathu Heenati, IR62, and IR65482-7-216-1-2 were consistently more resistant than TN1 (**Table 3**).

Table 3. Parameters of performance of *Nilaparvata lugens* from three colonies (following 20+ generations of selection) on 17 differential rice varieties. Mean plus standard error in brackets.

Rice variety	Biomass build up[1]				Plant weight loss/nymph biomass[2,4]				Eggs laid[3,4]			
	Colony				Colony				Colony			
	IR65482-4-136-2-2-SCPEC	IR62-SCPEC	TN1-Laguna		IR65482-4-136-2-2-SCPECA	IR62-SCPECB	TN1-LagunaA		IR65482-4-136-2-2-SCPECB	IR62-SCPECA	TN1-LagunaAB	
PTB33	3.91 (0.73)	2.41 (0.88)	2.53 (0.55)	a	-0.018 (0.03)	0.18 (0.02)	-0.11 (0.04)	abc	49 (7.25)	60.6 (7.63)	48.6 (6.23)	a
Babawee	5.93 (0.94)	6.17 (1.53)	2.66 (0.31)	a	-0.093 (0.05)	0.13 (0.02)	-0.26 (0.11)	Ab	75 (9.77)	64.6 (8.98)	86.6 (7.71)	abc
Rathu Heenati	0.22 (0.05)	4.75 (2.26)	0.81 (0.10)	a	-0.09 (0.02)	0.31 (0.10)	-0.19 (0.03)	abc	43 (0.62)	83 (8.93)	73.6 (4.03)	ab
Balamawee	6.03 (1.84)	11.20 (2.58)	7.72 (3.92)	a	0.14 (0.03)	0.34 (0.07)	0.10 (0.01)	abcd	62.8 (8.20)	40.6 (7.97)	62.2 (9.02)	a
IR62	44.02 (16.91)	11.91 (3.01)	10.12 (1.52)	a	0.08 (0.04)	0.05 (0.03)	-0.38 (0.06)	A	82.6 (3.26)	66.4 (10.37)	47.6 (4.88)	ab
IR65482-7-216-1-2	12.67 (2.38)	12.14 (2.82)	9.68 (1.73)	a	-0.03 (0.04)	-0.02 (0.05)	0.05 (0.01)	abc	75.8 (10.71)	89 (6.16)	93 (4.48)	abc
IR65482-4-136-2-2	7.95 (1.25)	19.46 (1.91)	93.44 (37.66)	ab	0.15 (0.02)	0.26 (0.04)	0.12 (0.33)	abcd	52.6 (7.13)	73.6 (5.38)	99 (8.63)	abc
IR40	80.55 (33.93)	8.62 (4.12)	163.040	abc	-0.07 (0.12)	0.31 (0.04)	0.18 (0.01)	abcd	109.6 (11.53)	61.4 (8.57)	47 (5.34)	abc
IR66	28.62 (10.89)	7.58 (2.24)	40.50 (15.38)	a	0.09 (0.03)	0.52 (0.14)	0.21 (0.06)	abcd	61.6 (5.38)	45.2 (7.99)	88.2 (11.15)	ab

Mudgo	28.98 (8.79)	34.65 (7.77)	8.67 (2.53)	ab	0.02 (0.01)	0.26 (0.01)	0.05 (0.01)	abcd	129.4 (6.15)	62.2 (5.44)	128.8 (11.45)	bcd
ASD7	19.92 (1.98)	25.26 (4.83)	13.22 (5.52)	a	0.26 (0.05)	0.14 (0.04)	0.17 (0.03)	abcd	136.2 (6.86)	61.6 (5.85)	87.8 (13.22)	abc
IR24	20.30 (5.32)	17.28 (2.18)	16.284	a	0.22 (0.02)	0.25 (0.03)	0.10 (0.03)	abcd	88.4 (9.29)	111.2 (9.61)	118.8 (11.19)	bcd
Swarnalata	28.00 (2.66)	6.73 (1.80)	27.13 (4.37)	a	0.25 (0.05)	0.39 (0.15)	0.51 (0.12)	D	133 (2.60)	86.6 (5.15)	67.4 (4.24)	abc
Chinsaba	22.22 (1.79)	13.72 (2.89)	31.48 (13.82)	a	0.11 (0.01)	0.27 (0.04)	0.12 (0.01)	abcd	137.4 (1.91)	105.4 (4.13)	118.8 (6.86)	cd
T65-NIL-*BPH25*	115.48 (64.99)	133.32 (57.66)	82.89 (39.75)	bc	0.20 (0.03)	0.38 (0.05)	0.17 (0.02)	abcd	83.4 (5.36)	71.6 (1.71)	56.8 (11.97)	ab
T65-NIL-*BPH26*	11.00 (2.54)	54.02 (21.06)	77.35 (29.01)	abc	0.12 (0.02)	0.43 (0.05)	0.18 (0.02)	abcd	103.2 (9.36)	92.8 (9.03)	121.8 (5.98)	bcd
TN1	100.70 (15.79)	133.36 (18.14)	147.060	c	0.42 (0.04)	0.45 (0.07)	0.18 (0.01)	D	174.4 (6.144)	112 (3.67)	173.2 (9.30)	d
F-colony[5]	0.755				7.018***				14.653***			
F-variety[5]	3.282***				5.159**				4.025***			
F-interaction	0.560				1.682*				1.018			
Error df	150				204				194			

1: Biomass build up by planthoppers standardized to final plant weight, lowercase letters indicate homogeneous groups (Tukey test: $P \leq 0.05$)

2: (Control plant dry weight – infested plant dry weight)/nymph dry weight, lowercase letters indicate homogeneous groups (Tukey test: $P \leq 0.05$)

3: Per plant (2 gravid females), lowercase letters indicate homogenous groups (Tukey test: $P \leq 0.05$)

4: Uppercase letters indicate homogenous colony groups based on fitness across all 17 varieties

5: DF colony = 2, DF variety = 16; * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ (univariate GLM)

4.0 Discussion

The large-scale deployment of planthopper resistant rice varieties in the 1970s and 1980s and resulting adaptation by the brown planthopper to these varieties has been well documented (Horgan, 2012 and references therein). The International Rice Research Institute (IRRI) played a pivotal role in screening for resistance among a huge collection (several 1000s) of rice varieties, identifying the first recognized resistance loci and transferring resistance from donor parents to high-yielding varieties such as IR24, IR36 and IR56 (Brar et al. 2009; Fujita et al. 2013). These varieties were distributed to national extension programs in Asia and have been widely planted (Khush and Virk, 2005). Currently, several national programs in Asia still deploy 'resistant' varieties based on early material acquired from IRRI: these mainly possess the *Bph1* and *bph2* genes that are now ineffective throughout the region (Fujita et al. 2013). For example, IR64 (*Bph1*) and derived varieties (i.e., Ciherang) are among the most popular varieties in Java (Indonesia) and IR64 and IR36 (*bph2*) are still widely planted in the Philippines (Launio et al. 2008). The only other planthopper resistance genes that have been deliberately deployed through IRRI are *Bph3* and *bph4*; however, adoption by farmers of varieties with these genes has been generally low (Brar et al. 2009; Peñalver Cruz et al. 2011). Other varieties (i.e., Inpari13 in Indonesia) with observed resistance to planthoppers have been deployed, but the underlying genetics of resistance in many of these varieties are poorly understood (Horgan, pers. obs.). Currently there are about 34 planthopper resistance genes available to rice breeders (Brar et al. 2009; Fujita et al. 2013). The vast majority of these genes have never been deployed in modern rice varieties.

Despite the limited deployment of planthopper resistance genes, a picture is emerging of low levels of resistance to planthoppers or the ineffectiveness of many genes in reducing planthopper damage (Alam and Cohen, 1998; Ketipearachchi et al. 1998; Myint et al. 2009a,b; Chen et al. 2011; Peñalver Cruz et al. 2011; this study). Throughout Asia,

planthoppers are virulent on varieties with *Bph1* and *bph2*. Furthermore, planthoppers virulent on varieties with *bph4* and *bph8* have also been identified (Myint et al. 2009a). Perhaps the most worrisome results have come from research conducted in Japan: planthoppers that migrated to Japan from China have been maintained as relict colonies (since 1969, 1989, and 1999) (Myint et al. 2009a). These planthoppers, when examined for their responses to resistance genes showed a gradual increase in the number of resistance genes that had been overcome: from zero (1969) to one (*Bph1* - 1989) to four (*Bph1*, *bph2*, *bph4*, *bph8* - 1999) without widespread deployment of many of these genes. Our results corroborate the results from Japan: Although TN1 was the most susceptible variety in our study, we found Philippine planthoppers able to feed and lay eggs on all 24 differential varieties. Only Rathu Heenati, PTB33 and IR62 - each with the *Bph3* gene - or Balamawee - with the *Bph9* gene - showed significant negative effects on planthopper fitness when compared against the susceptible controls IR22 or TN1. Planthoppers on some Babawee (*bph4*) and IR65482-4-136-2-2 (*Bph10*) rice plants had decreased fitness, but responses were highly variable. We suggest that these latter two varieties be considered as moderately resistant to Philippine planthoppers. Of significant note is the ineffectiveness of *Bph1*, *bph2*, *Bph6*, *bph8*, *Bph18*, *BPH25* and *BPH26*. The variety Yagyaw with the *Qbph3* and *Qbph9* genes was also ineffective. The results also indicate the likely absence of the *Bph3* gene in IR74. This variety is reputed to contain *Bph3*, but shows no effect against avirulent planthoppers (see also Peñalver Cruz et al. 2011).

The adaptation by planthoppers to a suite of genes that have never been widely deployed suggests that the 'Red Queen' approach to deployment of resistant rice varieties, based on the current understanding of planthopper resistance, is fundamentally flawed (Horgan, 2012). By this approach, breeders and geneticists sequentially deploy resistance genes replacing those genes, against which the target insect has adapted, by newer genes that

have never been previously deployed. However, our results suggest that planthopper adaptation to one particular gene may reduce the effectiveness of other genes. These may be genes located at the same or close position on a rice chromosome and that are likely to have similar functions. This may also include genes from wild rice parents, which may occur at a similar position in the wild parent as some native resistance gene in rice, but after hybridization now occurs at a different location in the introgressed breeding line. We examined how planthopper selection on a highly resistant variety such as PTB33 might affect the effectiveness of resistance in general. During our experiment, the selected colony was composed of planthoppers that had never experienced any variety but PTB33 for over 20 generations. At the time of the experiment the planthoppers were apparently not well adapted to PTB33. We regard evidence for adaptation as equal fitness of planthoppers on the resistant variety as on some standard susceptible control. However, the relative performance of our PTB33-adapted planthoppers on PTB33 was generally poor compared to performance by the same colonies on the susceptible controls. Nevertheless, relative biomass build-up on PTB33 was generally better for selected planthoppers (PTB33-SCPEC) than for non-selected planthoppers (IR22-SCPEC) and the performance rank was higher for selected planthoppers than non-selected planthoppers on the resistant varieties Balamawee and Rathu Heenati. This suggests that selection on PTB33 improved planthopper performance not only on lines with the same *Bph3* resistance gene, but also for a variety with an apparently unrelated gene – i.e. *Bph9*. Similarly, for a colony selected on IR62, we noted improved virulence on the same two varieties - Rathu Heenati and Balamawee. Previously, Peñalver Cruz et al. (2011) indicated that planthoppers adapted to IR62 (*Bph3*) were also more virulent on Babawee (*bph4*) than non-virulent planthoppers from a TN1 colony. *Bph3* and *bph4* are both located on the short arm of chromosome 6 and may represent the same resistance gene (Jairin et al. 2007; Peñalver Cruz et al. 2011; Fujita el al. 2013). Similarly, in a study by Ketipearachchi et al.

(1998) planthoppers adapted to *bph8* through selection on Thai collection 11, were also more virulent to Pokkali (*Bph9*) than were non-selected colonies. The location of *bph8* has not been mapped, but *Bph9* has been located on the long arm of chromosome 12. Therefore, our results suggest that planthopper adaptation to one gene can lead to reduced efficiency of resistance related to another gene, irrespective of gene location.

We hypothesized that planthoppers that had adapted to resistant varieties would have increased fitness on varieties with the same or closely related resistance genes. Our results using IR62-selected and PTB33-selected colonies, together with results from a study by Peñalver Cruz et al. (2011) support this hypothesis. Furthermore, the weak virulence response by planthoppers to selection on PTB33, a variety with several resistance genes, but relatively strong selection by the same planthoppers to feed on Rathu Heenati and Balamawee, suggests that gene pyramiding, though it may prolong the durability of resistance in a given variety, might not delay adaptation against individual genes in that same variety. We also hypothesized that planthoppers adapted to resistant varieties would experience fitness costs that reduce their ability to feed on unrelated or dissimilar varieties. We regard as evidence of fitness costs a decline in the ability of planthoppers to feed on any other varieties (irrespective of resistance genes and irrespective of possible increased fitness on other varieties). Our results generally do not support this hypothesis; however, we did note that planthoppers selected on PTB33 performed poorly on some of the most susceptible varieties, compared to their performance on the more resistant varieties. This was most apparent in the rank order of rice resistance against the PTB33-selected colony measured as population biomass build-up and suggests that planthopper adaptation mechanisms and their associated costs might shift virulence between groups of varieties or genes. Our results suggest that adaptation to *Bph3* possibly reduces planthopper virulence against varieties with *Bph1*, *bph2*, or *Bph6* genes. Experiments with the IR62-selected and IR65482-4-136-2-2-selected colonies

did not support the hypothesis since colonies generally performed equally well when exposed to varieties with different genes, irrespective of the varieties on which they had been selected. However, this hypothesis needs further examination since our selected colonies generally appeared not to be well adapted to the resistance genes for which they were selected.

Knowledge of the mechanisms of planthopper adaptation to resistant varieties would be helpful in determining potential fitness costs. However, the actual mechanisms of resistance underlying the resistance genes are not well understood, making determination of adaptation mechanisms also difficult (Ferrater et al. 2013; Fujita et al. 2013). The fast pace of adaptation to some resistance genes (i.e., *Bph1* and *bph2*) suggests that mechanisms other than directional selection may be involved. One idea is that endosymbiotic bacteria and yeasts might facilitate virulence adaptation (Chen et al. 2011). In general, however, the results of Ferrater et al. (Chapters 3 and 4) do not support a role for yeast-like endosymbionts in such adaptation. A more likely explanation, but one that has received little or no attention for planthoppers, is adaptation through epigenetic mechanisms.

The results of this study indicate that brown planthopper resistance genes available to rice breeders are more limited than might be suggested from the literature. For example, of 12 genes and 2 QTLs examined here, only three or four (*Bph3*, *bph4*, *Bph9* and possibly *Bph17*) reduce damage to rice plants by Philippine planthoppers. Nevertheless, one of these genes (*Bph3*) is highly durable and maintained a high ranking even against colonies selected on varieties with the *Bph10* or *Bph3* genes. The recognized high resistance from some of these genes might be their eventual downfall if national and international (because planthoppers migrate) rice breeding programs do not develop a common strategy to conserve resistance genes. Large-scale and continued deployment of resistance genes will increase selection for virulence. Pyramiding genes may not be a solution to prolonging gene life, since *Bph3* at least, seems to contribute in an additive manner to resistance in a naturally pyramided line

such as PTB33. Rice programs, therefore, need to limit deployment of resistant varieties (temporally and spatially) and reduce other crop management practices (high nitrogen use and prophylactic insecticide use) that might accelerate adaptation. Withdrawal from rice fields of varieties that are now ineffective (i.e, those with *Bph1* and *bph2* genes) might also relax selection for virulence against these genes and recover some of their value over time. This will occur if there are fitness costs to planthopper virulence – for which, however, currently there is only minor evidence. Resistance is an important component of the integrated management of rice brown planthoppers (Bottrell and Schoenly, 2012), but it should be better integrated into national planthopper management policies by making farmers and scientists aware of the need for the special management of resistant varieties (Teetes et al. 1994) and by increasing research attention to aspects of virulence evolution in the field.

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CHAPTER 7

General Discussion: Lessons learned and new perspectives on brown planthopper adaptation

Jedeliza B. Ferrater

1.0 Introduction

Throughout South and Southeast Asia, national and international programs exist to develop rice varieties with resistance to the brown planthopper, *Nilaparvata lugens* (Stål). To date more than 36 genes and major quantitative trait loci (QTL) for *N. lugens* resistance have been identified in rice (Fujita et al. 2013). However, *N. lugens* has been observed to rapidly adapt to resistant rice varieties in both the laboratory and field (Pathak and Heinrichs, 1982; Claridge and den Hollander, 1982; Alam and Cohen, 1998).The nature of adaptation by planthoppers to resistant rice varieties has been well documented from selection experiments (Pathak and Heinrichs, 1982; Claridge and den Hollander, 1982; Alam and Cohen, 1998). However, these experiments have been largely descriptive and generally only trace changes in planthopper fitness over successive generations to compare the relative durability of varieties or genes (Pathak and Heinrichs, 1982; Claridge and den Hollander, 1982), or the relative virulence of field collected populations (Alam and Cohen, 1998). Very few studies have examined the underlying mechanisms of adaptation in *N. lugens*. This is partly due to the lack of information about how resistance genes actually function (Horgan, 2009; Fujita et al. 2013), but also because of methodological difficulties (logistics and costs) inherent in selection and adaptation studies (see below). At the outset of this thesis several potential adaptation mechanisms were discussed, including directional selection, epigenetic shifts in planthopper gene functioning, and symbiont-mediated adaptation. This latter mechanism could refer to shifts in symbiont abundance, in the relative abundances of different symbiont

species, or in the functioning of symbionts (brought about by changes in symbiont genes and epigenetic changes in symbiont gene functioning). A small number of studies suggested that symbionts could play a role in adaptation by planthoppers to resistant rice (Lu et al. 2004; Tang et al. 2010; Chen et al. 2011). These preliminary studies each had similar methodological limitations that prompted a more thorough examination of the potential for symbionts to mediate adaptation.

2.0 Research on yeast-like symbionts and their potential role in adaptation

Adaptation by planthoppers to resistant rice varieties has been phenomenally rapid, and planthopper populations with virulence against several resistance genes are now widespread (Myint et al. 2009a,b; Peñalver Cruz et al. 2011; Fujita et al. 2013). Symbionts, a component of the internal flora of both planthoppers and leafhoppers (Ferrater et al. 2013; Wang et al. 2010; Tang et al. 2010), have recently been linked to variations in the outcome of rice-planthopper interactions (Lu et al. 2004; Tang et al 2010; Chen et al. 2011). Lu et al. (2004) demonstrated an initial reduction in yeast-like symbiont (YLS) abundance after *N. lugens* were switched between rice varieties [susceptible (TN1) to resistant (Mudgo or ASD7)], followed by a gradual increase in symbiont numbers over successive generations of selective rearing on the resistant varieties. Using the same rice varieties, Chen et al. (2011) examined brown planthopper colonies during two generations of selection (the 8th and 11th) and showed that YLS improved nymph performance in the 8th generation, but appeared to be a drain on the nymphs in the 11th generation (as shown by the higher performance of the aposymbiotic nymphs than the symbiotic nymphs in the 11th generation)(Chen et al. 2011). Furthermore, Tang et al. (2010) examined the bacterial 'symbionts' associated with planthoppers selected on TN1, Mudgo and ASD7, and found distinct bacterial communities between planthoppers reared on the three hosts. Chapter 2 of the present thesis discusses these results, but indicates that previous studies had some major flaws. Although the results

from each of these studies were novel and pointed to interesting adaptation mechanisms that could be potentially applied to combat the increasing virulence of planthoppers throughout Asia, each of the studies was pseudoreplicated. Pseudoreplication seriously limited the validity of previous research and calls for a more thorough examination of potential symbiont-mediated adaptation. Furthermore, each of the previous studies used the same three rice varieties, and therefore limited possibilities to demonstrate any diversity of symbiont responses. In the present thesis, steps were taken to improve methods and increase the external validity of the results. These included four main steps:

2.1 Proper replication of colonies during long term selection:

Pseudoreplication is common in selection studies; perhaps largely because of the costs involved in maintaining planthoppers or other insects during several generations. Initial studies, that proposed the existence and described the nature of planthopper biotypes were all unreplicated and therefore could not distinguish between divergence in selection as a result of populations being in isolation, and divergence as a result of populations reared on different host plants (Horgan, 2009). Because biotypes became a feature of resistance research in planthoppers on rice, this methodological shortcoming was carried over to recent studies. Of the several selection studies conducted using plant and leafhoppers on rice (Pathak and Heinrichs, 1982; Claridge and den Hollander, 1982), only Alam and Cohen (1998) and Vu et al. (2014) actually replicated their colonies.

In Chapter 3 of the present thesis, colonies with planthoppers initially collected at six locations were used as replicates. This replication improved the validity of our results because any significant trends in planthopper fitness or symbiont abundance were clearly a feature of the experimental treatments and not the test colonies. However, selection results in inbreeding and planthopper quality, particularly after severe bottlenecks such as those resulting from knock-down on resistant varieties, declines throughout selection. Therefore,

following selection, colonies with common feeding histories were combined to reduce inbreeding. These colonies were denoted as the South-Central Philippines Experimental Colony (SCPEC).

The SCPECs were used in the experiments in Chapter 4. Since the focus of the research was on the responses by planthoppers and symbionts during switching, the experiment was replicated using colonies with distinct feeding histories (one colony per natal host plant = 5 colonies). Significant trends, that were largely similar irrespective of colony or natal host plant clearly indicated that the symbionts played little role during switching.

In Chapters 5 and 6, the SCPECs were again used. The experiments in Chapter 5 were not replicated at the level of colony (only the IR62-selected colony was used) and therefore, do not have external validity, but refer only to the conditions of the experiment. This was partly because of the logistical difficulties in conducting the experiment. In Chapter 6 the entire experiment was replicated three times by using different SCPECs (IR62-, IR65482-, PTB33-selected colonies). Similar trends in increasing virulence pointed to a clear mechanism that was independent of the resistant varieties on which the planthoppers had been reared. The overall results indicated that selection for resistance produced planthoppers with a broader diet.

2.2 Managing and maintaining planthopper colonies

Previous research on planthopper and leafhopper adaptation to resistant rice varieties used very few insect colonies (normally one per host-plant treatment) that were maintained over several generations (usually < 15). Proper replication of colonies multiplies the number of colonies substantially; for example in Chapter 3 a total of 30 colonies were maintained during 20 generations to assess adaptation on 5 host plants. Maintaining so many colonies for so long is a major challenge. In Chapter 3, small plexiglass cages and soil-less media were used to rear the planthoppers. This had the advantage of reducing space in the insectary and

maintaining near-sterile conditions that aided in studying microbial activity (such as YLS densities). However, it may have contributed to instability during selection (with progeny sometimes appearing less adapted than their parents) and because populations went through bottlenecks, this would have increased inbreeding depression among the colonies. To counter this, populations were outbred in Chapters 4, 5 and 6. In future studies, larger populations should be maintained in larger cages and various strains selected for later admixing to avoid inbreeding.

2.3 Selecting plant materials for research:

Selection for *N. lugens* on resistant varieties has been conducted since the 1980s using Mudgo and ASD7 containing the *Bph1* and *bph2* resistance genes, respectively (Pathak and Heinrichs, 1982; Claridge and den Hollander, 1982). For over 20 years now, *Bph1* and *bph2* have not been effective against planthoppers in Asia. However, despite widespread planthopper adaptation, studies have continued to focus on these varieties (Lu et al. 2004; Ji et al. 2013; Xu et al. 2014; Tang et al. 2010; Chen et al. 2011). In the present thesis, three varieties with effective resistance against planthoppers were used (IR62, IR65482, PTB33). Furthermore, a susceptible variety (IR22) other than TN1 was used. These varieties were selected for experiments following preliminary screening. As indicated in Chapter 6, many published resistant varieties are no longer effective against planthoppers. Therefore, prior screening to select suitable varieties for selection/adaptation studies should be a standard procedure.

2.4 Standardization of symbiont abundance:

Some previous studies have not standardized for variability in planthopper weight. For example Lu et al. (2004) refer to changes in symbiont abundance during selection, without regard to the often severe decreases in body size that are observed among planthoppers on resistant rice plants. If body weight declines, but symbiont abundance

remains the same, then the function of the symbionts might be actually enhanced (greater symbiont biomass to insect biomass ratio). Therefore, standardization of insect body weight (referred to as symbiont density in this thesis) should be conducted during comparative studies of endosymbionts.

2.5 Manipulative experiments to address research hypotheses:

Manipulative experiments are required to address biological processes where proper hypothetico-deductive hypotheses are employed (Romesburg, 1981). Planthoppers selected on a single variety for several generations are good sources of material (insect host and its associated symbionts) that can be evaluated beyond descriptive (cause and effect) studies. Some studies have used such insect materials for later manipulative experiments with symbionts deliberately removed/reduced by generating aposymbiotic planthoppers and evaluated for their responses to different rice lines (Lu et al. 2004; Chen et al. 2011). In this thesis, we address processes by similarly manipulating symbiont densities (Chapter 4) and by selecting planthoppers on different varieties (Chapters 3, 5 and 6). Other manipulations included controlled switching of planthoppers with known virulence between varieties (Chapter 4), inducing responses by controlled exposure of rice plants to planthopper feeding (Chapter 5). These manipulative experiments were applied to address several research hypotheses. These hypotheses are outlined in the following sections and are evaluated based on results from the relevant experiments.

3.0 Hypothesis 1: Changes in yeast-like symbiont density mediate planthopper adaptation to resistant rice varieties

Based on previous studies, particularly the study by Lu et al. (2004), but also by Chen et al. (2011), we expected YLS to initially decline when planthoppers were first moved to resistant varieties, but to then gradually build-up and approach densities typical of susceptible varieties (i.e., TN1) and eventually level off when adaptation was complete. This pattern

would suggest a functional role for YLS in promoting survival and fitness of planthoppers; however, such patterns do not rule out the possibility that planthoppers first adapt to the host plant and that YLS simply recover numbers because of improved planthopper feeding ability. Nevertheless, such a pattern is necessary to support the hypothesis.

To examine the generality of such a pattern, in Chapter 3 planthopper populations from six sources were each exposed to four rice varieties (one susceptible and three resistant) over 20 generations. Contrary to expectations, the density of YLS was lowest on the most susceptible variety (IR22), which already indicated that the YLS density-mediated adaptation hypothesis was unlikely to be valid. Furthermore, YLS densities were largely similar on IR62 compared to TN1, and higher on PTB33 (which is highly resistant) compared to TN1. Only YLS densities on IR65482 approached the predicted pattern, but this was not enough to support the hypothesis as a general mechanism. In Chapter 3, slight reductions in YLS densities toward the end of selection on TN1, IR22 and IR62 may indicate that YLS have lower functional significance where varieties and environmental conditions are the same between generations. This possibility was further tested in Chapter 4 (hypothesis 2 below).

Apart from fitness monitoring during the course of selection, a closer look at feeding behaviour was taken in Chapter 3. Some behavioural changes during adaptation were noted and suggest that the nature of feeding behaviour is different for planthoppers on resistant and susceptible varieties, even after apparent adaptation. The type of honeydew excreted by the planthoppers indicated that on resistant varieties, planthoppers probe and feed more on the xylem compared to planthoppers on susceptible varieties – even when they had apparently adapted to the resistant host (i.e., after 20 generations). Xylem is largely considered nutrient deficient and is mostly water (Andersen et al. 1989). Xylem-feeding is thought to be related to water uptake (Spiller et al. 1990) and is a means for an insect to dilute its gut contents, and thus minimize dehydration (Pompon et al. 2010). For *N. lugens,* which is a typical phloem

feeder, xylem feeding is normally considered an indicator of host plant resistance (Yoshihara et al. 1980; He et al. 2010). The shift to xylem feeding among planthoppers on resistant varieties may be to dilute defensive compounds in the phloem of the resistant varieties. The mixed feeding found in this study is a novel finding for *N. lugens* subjected to long-term artificial selection on resistant rice varieties.

4.0 Hypothesis 2: The density of yeast-like symbionts facilitates host plant switching in planthoppers

Chen et al. (2011) found that symbionts increased the fitness of *N. lugens* on several rice host plants but may become a 'drain' on the insects where host varieties and environmental conditions are the same over several generations. These observations led to our second hypothesis that symbiont density has a role in the ability of *N. lugens* populations to switch between different rice varieties. Phenotypic differences between plants can determine feeding preferences in oligophagous and monophagous insects and can act as a barrier to switching by insects between plants of the same species (Mello and Silva-Filho, 2002). We predicted that if YLS density affected the capacity of planthoppers to switch between hosts, any decline in fitness of aposymbiotic planthoppers compared to symbiotic hoppers would be greatest when the planthoppers were switched to a new variety (exposed plants) relative to their performance when switched between two plants of the same variety (natal hosts). We further predicted that if symbionts had become a drain for the planthoppers, particularly where host varieties remained constant (i.e., no switching), aposymbiotic planthoppers will perform better than symbiotic planthoppers.

These predictions were examined by switching adapted planthoppers (selected for 20+ generations on a single natal host) to new varieties (exposed plants) under normal YLS densities (symbiotic) and after reduction of YLS densities by heat treatment (aposymbiotic). In addition, under the same conditions, we tested whether symbionts had become a drain for

the hoppers during successive generations of selection and during switching by comparing fitness of both aposymbiotic and symbiotic planthoppers that were either switched between rice varieties or maintained on the same varieties.

The results showed that there was a reduction in planthopper fitness (nymph weight) when YLS densities were depleted through heat treatment indicating that the YLS were not a drain on the host insect. However, compared to untreated symbiotic planthoppers, the depletion of symbionts did not generally change the relative fitness of planthoppers (each reared on a single natal host plant) when switched to feed on a range of different lines (exposed plants). Proportional weight changes on exposed plants relative to natal plants for symbiotic and aposymbiotic nymphs were generally similar, indicating that removal of the majority of symbionts by heat treatment influenced body weight but not the relative capacity of nymphs to feed on different plants.

5.0 Hypothesis 3 (virulence acquisition hypothesis): Virulence is acquired indirectly through horizontal transmission of virulence-promoting factors between insects feeding on the same plant

Planthopper virulence adaptation is usually regarded as a gradual shift in the ability of planthoppers to feed and oviposit on resistant varieties following continuous selection on an abundant, resistant host (Alam and Cohen, 1998; Ferrater et al. 2015). However, the rate of planthopper adaptation to resistant host plants suggests that other, more rapid mechanisms for the acquisition of virulence may exist. For example, insect herbivores secrete molecules called elicitors present in insect saliva that either induce or suppress defenses in host plants (Turlings et al. 1990, 1993; Musser et al. 2002; Louis et al. 2013). Furthermore, Wang et al. (2008) noted bacteria-like organisms in the leaf sheets of rice associated with planthopper feeding. These observations point to an alternative hypothesis to explain rapid virulence acquisition. Chapter 5 tests the hypothesis that planthoppers may gain virulence against

resistant varieties by acquiring virulence factors (i.e., as bacteria and proteins in the oral secretions) indirectly through host plants on which virulent planthoppers contemporaneously feed or has previously fed. If this hypothesis is correct, then virulence could be acquired by avirulent planthoppers when these ingest virulence promoting materials (bacteria, etc.) derived from virulent conspecifics at shared feeding sites.

In Chapter 5, optimal conditions were created for feeding-associated contamination of a tolerant feeding plant (Triveni) by a planthopper population with virulence against a resistant variety (IR62). It was expected that virulence-promoting factors (i.e., BLOs) could be picked up by avirulent planthoppers that later feed on the same plants. Avirulent planthopper populations were therefore allowed to feed on the Triveni plants and later examined for improvements in fitness on the same and novel varieties including the resistant variety IR62.

Results revealed that planthoppers attained highest weights on those plants on which virulent planthoppers had previously fed which suggests that feeding by the virulent planthoppers (IR62-resistance adapted) best facilitated subsequent planthopper feeding on the same plant. In one of two cases, we have observed that virulence can be acquired by avirulent planthoppers which shared feeding sites with virulent planthoppers. The idea that horizontally transmitted virulence may be passed to progeny was also examined: progeny attained similar weights and excreted similar honeydew irrespective of the feeding history of their parents (i.e., whether these shared feeding sites with virulent planthoppers) in the first run of the experiment, but showed improved feeding on IR62 in the second run. These results suggest that feeding by mixed virulent-avirulent populations may accelerate adaptation by *N. lugens* to resistant rice varieties.

6.0 Hypothesis 4: Planthoppers selected on resistant varieties have increased fitness on other resistant varieties with the same or closely related resistance genes.

Of the 36 identified genes and quantitative trait loci expressing resistance against *N. lugens* (Fujita el at. 2013), only a few are currently effective in reducing planthopper fitness below levels observed on susceptible varieties (Horgan and Ramal, unpublished). However, some of these genes have not been widely deployed in traditional or modern rice varieties such that planthoppers have not had prior exposure to the genes/varieties to which they are apparently adapted. These observations suggest that planthoppers adapted to a single widespread, resistant variety may have increased fitness on varieties having the same or closely related resistance genes. Therefore, Chapter 6 tested this hypothesis by examining virulence of adapted planthoppers against a range of rice varieties. It was predicted that *N. lugens* selected to feed on resistant varieties would have a generally improved ability to feed and develop on varieties previously noted for their resistance.

The results in Chapter 6 indicated that the planthoppers were able to feed and lay eggs on all the varieties tested. Planthoppers that were selected on resistant varieties with the *Bph3* gene had increased fitness on varieties with the same (Rathu Heenati) or closely related (Balamawee – *Bph9*) genes, albeit with little apparent improvement on the natal host IR62.The adaptation by planthoppers to a range of genes that have never been widely deployed suggests that the established approach to deployment of resistant rice varieties, i.e. that breeders and geneticists sequentially deploy resistance genes replacing those genes, against which the target insect has adapted, needs to be reassessed.

7.0 Hypothesis 5: Planthoppers adapted to resistant varieties are subject to fitness costs that reduce their ability to feed on unrelated or dissimilar varieties.

This hypothesis is a corollary of the previous hypothesis, but the two hypotheses are not mutually exclusive. Both plant defence and insect adaptation involve a metabolic cost (Gatehouse, 2002). This metabolic cost can be expressed as a trade-off that limits performance of adapted populations on other hosts (Fellous et al. 2014). In Chapter 6, it was predicted that planthoppers selected to feed on resistant rice varieties might have a lower ability to successfully feed on dissimilar varieties. This hypothesis was tested by examining the relative fitness of planthoppers from colonies that had been selected for several generations on resistant varieties, when these were allowed to feed or oviposit on a range of other resistant varieties. However, there was no strong evidence for such fitness costs among three colonies that were selected on resistant varieties in the present study.

8.0 Recommendations for future research

This thesis has largely discounted the role of YLS in adaptation by planthoppers to resistant rice varieties. However, the thesis has focused mainly on the role of YLS density during adaptation and not on other factors including YLS community composition, or YLS functional efficiency. Other factors of YLS should therefore be considered in future research. Furthermore, this thesis limited the focus on YLS and did not consider the role of other endosymbionts such as bacteria. The research focus should now be shifted to the **bacterial symbionts**. Although several studies have explored the impact of microbes on the metabolism of the host, the properties of the community as a whole cannot be explained just by characterizing the component parts, i.e. bacteria, fungi (Relman, 2008). Therefore, microbiota should be studied using holistic approaches to gain insights to all exerted functions and activities (Booijink, 2009). For this purpose, the so-called meta-“omics”

approaches such as metatranscriptomics, metaproteomics, metagenomics and metametabolomics are suitable, as these focus on profiling microbial activity (Xu, 2010).

The term **metagenomics** refers to genomic analysis of a community by combining the comprehensive analysis of an organism's genetic material (genomics) with the separate analyses of a set of related data (meta-analysis). In addition, metagenomics provide researchers with genome catalogues that are representative for the microbial diversity (Handelsman, 2004). Metagenomics allows investigation of microorganisms that cannot be cultured in the laboratory (Schloss and Handelsman, 2005; Tringe and Rubin, 2005; Kimura, 2006; Brune, 2007; Eisen, 2007; Hoff et al. 2008; Sleator et al. 2008; Wooley et al. 2010). Metagenomic approaches have been used to assess bacterial communities of planthoppers on different rice varieties and are currently being applied to look at bacterial endosymbiont communities in leafhoppers selected on near-isogenic rice lines at IRRI (Srinivasan, Oliva and Horgan, unpublished).

A simpler way to characterize the role of bacterial symbionts in *N. lugens* adaptation to resistant varieties is the production of **bacteria-free planthoppers** using different sets of antibiotics. In this method, the issue of heat affecting the insects can be ruled out. When bacteria-free insects are generated, their impact on *N. lugens* host-plant utilization could be determined. In many aphid species, bacterial symbionts affect fitness and influence host insect phenotypes (Barbosa et al. 1991; Vega and Blackwell, 2005; Colvin et al. 2006). In addition, recent researches suggest that secondary bacterial symbionts in insects act as a mechanism for horizontal genetic exchange among hosts, facilitating adaptation to new ecological niches (Barr et al. 2010; Oliver et al. 2010; White, 2013).

Behavioral adaptation by *N. lugens* during selection on resistant varieties suggests that the insects feed on the xylem even after adaptation was achieved (mix-feeding). It is recommended to use **Electrical Penetration Graph (EPG)** technology to provide further

details of behavioural responses of adapted and non-adapted brown planthoppers during host-plant switching and similarly when using aposymbiotic planthoppers. EPG will give more detailed quantifiable behavioural observations that can be related to fitness traits such as nymph weight or development times; however, EPG studies should not be conducted in isolation without related fitness measures.

Mix-feeding on xylem and phloem by *N. lugens* suggests that feeding deterrents/metabolites are present in the phloem of resistant varieties. However, very little information is available regarding these metabolites. It would be useful to **identify resistance factors** (i.e., chemicals/enzymes produced by the rice plants) and what substances the insects or their symbionts produce to neutralize these compounds in the virulent planthoppers.

It has recently also become clear that insect adaptation to its food plants is not necessarily only dependent on genetic variation at the DNA-sequence level (Jablonka and Lamb, 2006). **Epigenetic processes** are capable of yielding phenotypes that can be transmitted to subsequent generations of cells or organisms (Jablonka and Raz, 2009) and could underlie adaptations by planthoppers or their symbionts to resistant rice varieties. Currently, there is no convincing information on the functioning of any resistance genes against planthoppers. Once resistance gene functioning has been elucidated (Kohli and Vandegehuchte, personal communications), and planthopper populations that are affected by these genes are identified, only then can epigenetics be evaluated as a factor in adaptation.

9.0 Conclusions

The brown planthopper can adapt to resistant rice varieties that possess different resistance genes and mechanisms. In the experiments described in this thesis, adaptation was not particularly rapid and, possibly because of the rearing conditions used in the experiments, adaptation was not stable (Chapter 3). Nevertheless, planthoppers selected on resistant lines, had clear differences in YLS densities and in their ability to feed on different rice hosts.

Patterns in YLS density during selection suggested that density plays no significant role in adaptation (Chapter 3); furthermore, manipulation of YLS during switching experiments suggested that YLS do not mediate host plant switching in planthoppers (Chapter 4), although they do play a significant role in planthopper nutrition and survival that is independent of variety (Chapter 4). Virulence is a complex phenomenon and planthoppers with different levels of virulence affect the host plants differently. The most virulent planthoppers in one experiment (Chapter 5), appeared to suppress rice defences to a greater extent than non-virulent planthoppers, and this was mediated through the host plant. Adaptation by planthoppers continues to be a major concern for rice breeders and several formerly resistant rice varieties appeared to have lost their resistance in recent years (Chapter 6). Further research on adaptation mechanisms may help prolong resistance durability in rice fields.

SUMMARY

Adaptation of the brown planthopper, *Nilaparvata lugens* (Stål), to resistant rice varieties

This thesis examined adaptation by the brown planthopper, *Nilaparvata lugens* (Stål) to resistant rice varieties. It also addressed the potential role of yeast-like symbionts in feeding adaptation by the planthopper. Although not as well studied as the aphid-symbiont system, a few studies have implicated yeast-like symbionts in *N. lugens* adaptation. This thesis centred on a selection experiment conducted over 20 generations of planthoppers on 1 susceptible rice and 3 resistant varieties (Chapter 3). *Nilaparvata lugens* adapted to resistant host plants as indicated by increased egg-laying and adult weight. Xylem feeding was noted as a possible behavioural adaptation of *N. lugens* for feeding on resistant varieties.

Chapter 2 reviews the literature and examines the possibilities for symbiont-mediated adaptation by planthoppers to resistant rice varieties. The chapter indicates that certain feeding-related resistance mechanisms could directly affect symbionts and therefore, that symbionts could play a role in overcoming such defences. Furthermore, the chapter highlights knowledge gaps and methodological limitations in previous studies and developed hypotheses to be tested in the thesis.

Chapter 3 tests the hypothesis that YLS density is associated with virulence of *N. lugens*. However, it was found that YLS density has no consistent trends when planthoppers were reared on resistant rice varieties continuously for several generations. There were a few cases where a decline was observed in YLS density towards the end of selection which suggested that YLS may have a role in helping planthoppers cope with environmental change

and are not required by planthoppers in stable environments (i.e., when feeding continuously on a single variety over several generations).

Chapter 4 examined the role of YLS in host-plant switching where adapted planthoppers on a single natal host were transferred to new varieties under normal YLS densities (symbiotic) and after reduction of YLS densities by heat treatment (aposymbiotic). Results showed that there was a reduction in planthopper fitness (nymph weight) when YLS densities were reduced which indicates that the YLS were not a drain on the host insect. Proportional weight changes on exposed plants relative to natal plants for symbiotic and aposymbiotic nymphs were generally the same, indicating that removal of the majority of symbionts by heat treatment influenced body weight but not the relative capacity of nymphs to feed on different plants.

Chapter 5 examined the hypothesis that virulence is acquired indirectly by horizontal transmission of virulence-promoting factors between insects feeding on the same plant. Optimal conditions for feeding-associated contamination of a tolerant feeding plant by a planthopper population with virulence against a resistant variety where created. Avirulent planthoppers were later allowed to feed on the plants and potentially pick up resistance factors circulating in plant tissues after virulent planthoppers have fed. Results revealed that planthoppers attained highest weights on those plants on which virulent planthoppers had previously fed which suggests that feeding by the virulent planthoppers best facilitated subsequent planthopper feeding on the same plant. Results also suggest that feeding by mixed virulent-avirulent populations may accelerate adaptation by *N. lugens* to resistant rice varieties.

The results from Chapter 6 indicate that planthoppers were able to feed and lay eggs on a range of resistant rice varieties. Planthoppers that were selected on resistant varieties

with the *Bph3* gene had increased fitness on varieties with the same or closely related resistance genes. This supports the hypothesis that planthoppers selected on resistant varieties have increased fitness on other resistant varieties with the same or closely related resistance genes.This chapter also examines whether planthoppers adapted to resistant varieties demonstrated fitness costs that reduce their ability to feed on unrelated or dissimilar varieties. However, there was no strong evidence for such fitness costs among the insect colonies that were selected on the resistant varieties.The adaptation by planthoppers to a range of genes that have never been widely deployed suggests that the current approach to deployment of resistant rice varieties needs to be reassessed.

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LIST OF PUBLICATIONS

Ferrater JB, Naredo AI, Almazan MLP, de Jong PW, Dicke M, Horgan FG (2015) Varied responses by yeast-like symbionts during virulence adaptation in a monophagous phloem-feeding insect. Arthropod-Plant Interactions 9:215-224.

Ferrater JB, de Jong PW, Dicke M, Chen YH, Horgan FG (2013) Symbiont-mediated adaptation by planthoppers and leafhoppers to resistant rice varieties. Arthropod-Plant Interactions 7:591-605.

Ferrater JB, Horgan FG (submitted for publication) Do yeast-like symbionts facilitate host switching in a monophagous phloem-feeding insect? A test with planthoppers on rice.

Ferrater JB, Horgan FG (submitted for publication) Can virulence be acquired by *Nilaparvata lugens* from conspecifics at shared feeding sites?

CURRICULUM VITAE

Jedeliza Busgano Ferrater was born on July 21, 1975 in General Santos City, Philippines. In 1996, she completed her Bachelor's degree in Biology, cum laude, from Mindanao State University-General Santos. Prior to her graduation at university, she was hired as a lecturer at the Department of Biology and as a researcher to investigate the massive fish kills in Mindanao's Lake Sebu in 1997. Part of her research task was to culture nitrifying and denitrifying bacteria that will enhance microbial nitrogen cycling in the lake, reducing nitrogenous pollutants that caused the fish kills. This research inspired her to pursue a Master's degree in Microbiology at the University of the Philippines-Los Baños (UPLB), more than 1000 kilometers away from her hometown. Initially, she supported her master's degree as a part-time microbiologist at a nearby mineral water bottling company and as a food server in a Los Baños cafeteria. Within the first year of her MSc course, she was granted a full scholarship from the Philippine government's Department of Science and Technology. Before completing her master's degree, she was employed as a research associate at the Institute of Plant Breeding-UPLB where she was trained in molecular biology, characterizing the genetic diversity of bacteria, fungi and viruses of agricultural crops. Her master's thesis was "Analysis of the genetic diversity of *Cercospora canescens* (Ellis and Martin), the causal fungus of mungbean leafspot". Her laboratory skills and experiences in molecular techniques led her to a job at the International Rice Research Institute in 2005 where she developed microsatellite markers for the brown planthopper, *Nilaparvata lugens* (Stål). She pursued her PhD at Wageningen University in 2010 through the support of the Global Rice Science Scholarships (sponsored by Du-Pont Pioneer Overseas Corporation and International Rice Research Institute) studying the "Adaptation of the brown planthopper, *Nilaparvata lugens* (Stål), on resistant rice varieties". She is a fulltime Entomologist at East-West Seed Philippines since January 2015 working on various East-West and Dutch collaborative projects on insect-host plant resistance.

Publications

Ferrater JB, Naredo AI, Almazan MLP, de Jong PW, Dicke M, Horgan FG (2015) Varied responses by yeast-like symbionts during virulence adaptation in a monophagous phloem-feeding insect. Arthropod-Plant Interactions 9:215-224.

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PE&RC Training and Education Statement

With the training and education activities listed below the PhD candidate has complied with the requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)

Review of literature (6 ECTS)

- Symbiont-mediated adaptation by planthoppers and leafhoppers to resistant rice varieties (2011)

Writing of project proposal (4.5 ECTS)

- Adaptation of the brown *planthopper, Nilaparvata lugens* Stål to resistant rice varieties

Post-graduate courses (2.7 ECTS)

- Brown planthopper symbiont dynamics; Zheijiang Academy of Agricultural Sciences, China (2009)
- Linear models (2010)
- Mixed linear models (2010)

Deficiency, refresh, brush-up courses (7.5 ECTS)

- Insect-plant interactions; Laboratory of Entomology, WUR (2010)
- Basic statistics (2010)

Competence strengthening / skills courses (7.8 ECTS)

- Writing a research article for international publication; University of Adelaide, Australia (2011)
- Graduate studies for leadership in rice research; KU Leuven University, Belgium (2011)
- Rice: research to production course; Cornell University and National Science Foundation, USA (2011)

PE&RC Annual meetings, seminars and the PE&RC weekend (1.2 ECTS)

- PE&RC Day (2010)
- PE&RC Weekend (2010)

Discussion groups / local seminars / other scientific meetings (9.6 ECTS)

- IRRI 1st Young scientists conference; co-organizer (2012)
- Insect-host plant resistance meeting; IRRI (2010-2014)
- PhD Lunch meetings; WUR (2010)
- Insect-plant interactions lunch meetings; WUR (2010)
- Netherlands Annual Ecology Meeting; Lunteren (2010)
- Wiki-workshop in the framework of Arthropod symbioses: from fundamental studies to pest and disease management; European Commission on Science and Technology, Zürich, Switzerland (2010)

International symposia, workshops and conferences (4.7 ECTS)

- Insect and Virus Resistance: an international student symposium; main organizer and oral presentation; IRRI and Kyushu University, Japan and IRRI, Philippines (2011)
- XXIV International Congress of Entomology; oral presentation; South Korea (2012)

Supervision of 1 MSc student

- Can the virulence of the adapted brown planthoppers overcome the available resistance genes in rice?

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