EFFECTS OF THERMAL STRESS ON THE BROWN PLANTHOPPER NILAPARVATA LUGENS (STAL)

by

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Abstract

This study investigated the effects of heat stress on the survival, mobility, acclimation ability, development, reproduction and feeding behaviour of the brown planthopper *Nilaparvata lugens*. The critical information derived from the heat tolerance studies indicate that some first instar nymphs become immobilized by heat stress at around 30°C and among the more heat tolerant adult stage, no insects were capable of coordinated movement at 38°C. There was no recovery after entry into heat coma, at temperatures around 38°C for nymphs and 42-43°C for adults. At 41.8° and 42.5°C respectively, approximately 50% of nymphs and adults are killed. In a comparison of the acclimation responses between nymphs and adults reared at 23°C and acclimated at either 15 or 30°C, the data indicate that increases in cold tolerance were greater than heat tolerance, and that acclimation over a generation compared with a single life stage increases tolerance across the thermal spectrum.

The temperatures that kill around 50% of nymphs and adults also exert negative effects on development and longevity. The same exposures also lower fecundity and extend egg development time through a combination of mating groups, in which the greatest effects occur when both males and females have experienced sub-lethal heat stress. Likewise, exposure to their ULT₅₀ reduced feeding activity in both life stages of *N. lugens*. The amount of honeydew excreted by females and males in the treated nymph and adult groups were 3-4x and 2-3x lower than in the equivalent control groups. Overall, sub-lethal heat stress extended egg development time, inhibited nymphal development, lowered fecundity and reduced feeding activity.
This thesis is dedicated to my deceased parents

“Whatever the mind can conceive and believe it can achieve.”

Napolean Hill quote

Quoted in ‘Coming from Liberia’ by Ezike II, E.C. (2008)
Preface

This thesis is submitted for the degree of Doctoral of Philosophy at the University of Birmingham. All experiments were under the supervision of Professor Jeff Bale and carried out in the School of Biosciences from June 2009 to June 2012. This research is totally original and contains nothing that is the outcome of collaboration except where appropriately referenced. No part of this thesis has been submitted for any other degree, diploma or any other qualification. It does not exceed 50,000 words in length.

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- Piyaphongkul J., Pritchard J. and Bale J.S. Effects of acclimation on the thermal tolerance of the brown planthopper *Nilaparvata lugens* (Stål).
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• Piyaphongkul J., Pritchard J. and Bale J.S. Temperature calibration of system used to measure insect activity thresholds.

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**Table of contents**

List of Figures ........................................................................................................xiii

List of Tables..........................................................................................................xix

**Chapter 1 General introduction...........................................................................1**

1.1 Climate change.................................................................................................3

1.2 Impacts of climate change on insects...............................................................9

1.3 Thermal biology and insect physiology............................................................11

1.3.1 Background....................................................................................................12

1.3.2 Thermoregulation in insects.........................................................................14

1.3.3 Temperature tolerance..................................................................................19

1.4 Ecology and biology of the brown planthopper, *Nilaparvata lugens*................22

1.4.1 Development and life cycle..........................................................................23

1.4.2 Feeding behaviour.......................................................................................24

1.4.3 Migratory behaviour and distribution.........................................................25

1.4.4 Outbreaks and economic impacts...............................................................28

1.5 Objectives.........................................................................................................29
Chapter 2 Temperature calibration of system used to measure insect activity thresholds...30

2.1 Abstract.................................................................................................................30

2.2 Introduction...........................................................................................................31

2.3 Material and methods..........................................................................................33

2.4 Results..................................................................................................................36

2.5 Discussion and conclusions..................................................................................41

Chapter 3 Relationship between body size and dynamics of thermal equilibrium in insects........................................................................................................... 45

3.1 Abstract..................................................................................................................45

3.2 Introduction.............................................................................................................46

3.3 Material and methods...........................................................................................49

3.3.1 Insect materials..................................................................................................49

3.3.2 Measurement of surface-to-volume ratio.......................................................50

3.3.3 Measurement of $T_b$ and time required to reach thermal equilibrium...........50
Chapter 4 Upper thermal thresholds of the brown planthopper *Nilaparvata lugens* ........65

4.1 Abstract .............................................................................................................65

4.2 Introduction ......................................................................................................66

4.3 Material and methods ......................................................................................68

4.3.1 Insect cultures .............................................................................................68

4.3.2 Determination of $CT_{\text{max}}$ and HCT ..................................................69

4.3.3 Determination of ULT ................................................................................70

4.4 Results .............................................................................................................71

4.4.1 $CT_{\text{max}}$ and HCT ....................................................................................71
Chapter 6 Effects of heat stress on the development and fecundity of the brown planthopper *Nilaparvata lugens* ................................................................. 111

6.1 Abstract ................................................................. 111

6.2 Introduction ........................................................... 112

6.3 Material and methods ................................................. 116

6.3.1 Insect materials ..................................................... 116

6.3.2 Effect of sub-lethal high temperatures on development and longevity .............. 116

6.3.3 Effects of sub-lethal high temperatures on fecundity ........................................ 117

6.4 Results ................................................................. 119

6.4.1 Effect of sub-lethal high temperatures on development and longevity .............. 119

6.4.2 Effect of sub-lethal high temperatures on fecundity ........................................ 122

6.5 Discussion and conclusions ........................................... 126

Chapter 7 Influence of short exposure to high temperatures on feeding activity of the brown planthopper *Nilaparvata lugens* ................................................................. 132

7.1 Abstract ................................................................. 132

7.2 Introduction ........................................................... 133
Chapter 7 Material and methods .................................................................................................. 137

7.3 Material and methods .................................................................................................. 137

7.3.1 Plant .................................................................................................................. 137

7.3.2 Insects .............................................................................................................. 138

7.3.3 Honeydew collection and analysis ....................................................................... 138

7.4 Results .................................................................................................................... 140

7.4.1 Effects of sub-lethal high temperature exposure on feeding activity in nymphs ... 140

7.4.2 Effects of sub-lethal high temperature exposure on feeding activity in adults ..... 143

7.5 Discussion and conclusions ..................................................................................... 147

Chapter 8 General conclusions .......................................................................................... 152

8.1 Upper thermal limits of *N. lugens* in natural environment ....................................... 154

8.2 *Nilaparvata lugens* has less ability to increase heat tolerance than cold tolerance ... 155

8.3 Sub-lethal heat stress impedes development and lowers fecundity in *N. lugens* ... 156

8.4 Exposure of *N. lugens* at their ULT<sub>50</sub> reduces feeding activity ..................... 157

8.5 Future research ........................................................................................................ 159

References ...................................................................................................................... 160
List of Figures

**Figure 1.1** Components and interactions of the global climate system (from Ahlonsou et al., 2001).................................................................4

**Figure 1.2** Hypothetical performance curve of an insect as a function of body temperature, showing the 80% performance breadth ($B_{80}$), $CT_{min}$ and $CT_{max}$ (from Chown and Nicolson 2004).................................................................13

**Figure 1.3** Mechanisms of heat gain and loss in insects (adapted from Wharton, 2002)........16

**Figure 1.4** The zeroth law of thermodynamics; two objects (Y and X) that are independently in thermal equilibrium with a third object (Z), are also in thermal equilibrium with each other.................................................................18

**Figure 1.5** Responses to temperature in a hypothetical organism (adapted from Wharton, 2002; Chown and Nicolson 2004).................................22

**Figure 1.6** Distribution of *N. lugens* in Asia (Khush, 1979).................................26

**Figure 2.1** Aluminium block system for measuring insect activity thresholds; A, route for thermocouple to arena; B, observation arena; C, circulation channels for fluid from the alcohol bath (from Hazell et al., 2008).................................................................33

**Figure 2.2** Dimensions of arenas used to measure activity thresholds; 1-5 in Figure 2.2C indicate the positions of the five thermocouples in each arena.................................34
Figure 2.3 Relationship between arena surface temperatures in relation to the reference temperature in arenas of three sizes. A = small, B = medium and C= large. Measurement locations 1-5 in each arena are as coded in the figure………………………………………………36

Figure 2.4 Relationship between arena ambient temperatures in relation to the reference temperature in arenas of three sizes. A = small, B = medium and C= large. Measurement locations 1-5 in each arena are as coded in the figure………………………………………………………………………………37

Figure 2.5 Calibration graphs and equations for arena surface temperature and reference temperature in arenas of three sizes. For the small arena (A), $Y = 0.8322X + 3.8975$ ($R^2 = 0.997$), medium arena (B), $Y= 0.8072X + 4.3698$ ($R^2 = 0.992$) and large arena (C), $Y = 0.7672X + 5.0365$ ($R^2 = 0.978$)…………………………………………………………………………….39

Figure 2.6 Calibration graphs and equations for arena ambient temperature and reference temperature in arenas of three sizes. For the small arena (A), $Y = 0.7739X + 5.3846$ ($R^2 = 0.992$), medium arena (B), $Y= 0.7482X + 5.9242$ ($R^2 = 0.978$) and large arena (C), $Y = 0.7484X + 5.2351$ ($R^2 = 0.989$)………………………………………………………………………………40

Figure 2.7 Illustration of heat transfer effects in experiments to assess high (A) and low (B) activity thresholds in insects. $\Delta T$ is temperature difference between the arena and surrounding environment………………………………………………………………………………43

Figure 3.1 Experimental design for measuring body temperatures of insects when exposed in A, plastic tubes within glass tubes, and B, direct exposure within glass tubes…………………………….52
Figure 3.2 Mean surface-to-volume ratio (± SE) in *L. bryoniae*, *N. lugens* and *C. vicina*. Mean values with the same letter are not significantly different (p ≤ 0.05); n = 50 for each species.

Figure 3.3 Effect of insect body size on mean lag times (± SE) when exposed in plastic tubes within glass tubes (A) and in glass tubes (B). White bars represent *L. bryoniae*, black bars *N. lugens*, cross-hatch bars winged *C. vicina* and dotted bars wingless *C. vicina*. At 0.1°C min⁻¹ mean values with the same lower-case letter are not significantly different (p ≤ 0.05); at 0.5°C min⁻¹ mean values with the same lower-case letter followed by the same number are not significantly different (p ≤ 0.05) and at 1.0°C min⁻¹ mean values with the same capital letter are not significantly different (p ≤ 0.05). N = 30 at each ramping rate.

Figure 3.4 Effect of ramping rates on the mean lag time (± SE) when exposed in plastic tubes within glass tubes (A) and in glass tubes (B). White bars represent a ramping rate of 0.1°C min⁻¹, black bars 0.5°C min⁻¹ and cross-hatch bars 1.0°C min⁻¹. At 0.1°C min⁻¹ mean values with the same lower-case letter are not significantly different (p ≤ 0.05); at 0.5°C min⁻¹ mean values with the same lower-case letter followed by the same number are not significantly different (p ≤ 0.05) and at 1.0°C min⁻¹ mean values with the same capital letter are not significantly different (p ≤ 0.05). N = 30 at each ramping rate.

Figure 3.5 Effect of type of exposure container on mean lag times (± SE) at ramping rates of 0.1 (A), 0.5 (B) and 1.0°C min⁻¹ (C). White bars represent experiments carried out in plastic tubes within glass tubes and grey bars in glass tubes. Mean values with the same lower case letter (*L. bryoniae*), lowercase letter followed by the same number (*N. lugens*), capital letter (winged *C. vicina*).
vicina) and capital letter followed by the same number (wingless C. vicina) are not significantly different (p ≤ 0.05). N = 30 for each species in each type of container.................................59

**Figure 4.1** Thermal activity thresholds of different life cycle stages and sexes of *N. lugens*. Mean (± SE) CT_{max} (A) and HCT (B). Mean values with the same letter are not significantly different (p ≤ 0.05); n = 20 for first instar nymphs, adult females and males...........................................72

**Figure 4.2** Temperature range of thermal activity thresholds of different life cycle stages and sexes of *N. lugens*. Changes in the CT_{max} (A) and HCT (B) for first instar nymphs (white bars), adult females (cross-hatch bars), and adult males (black bars); n = 20 for each life cycle stage………………………………………………………………………………………………73

**Figure 4.3** Mean (± SE) ULT_{50} of first instar nymphs and adults of *N. lugens*. Mean values with the same letter are not significantly different (p ≤ 0.05); n = 50 at each exposure temperature................................................................................................................................................74

**Figure 5.1** CT_{min} of *N. lugens* acclimated at 15°C (white bars), 23°C (black bars) and 30°C (cross-hatch bars. Mean CT_{min} of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter within each graph frame are not significantly different (p ≤ 0.05); n = 20 for each life cycle stage.................................................................91

**Figure 5.2** Chill coma temperature (CCT) of *N. lugens* acclimated at 15 (white bars), 23 (black bars) and 30°C (cross-hatch bars). Mean CCT of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter are not significantly different (p ≤ 0.05); n = 20 for each life cycle stage..................................................................................................................92
**Figure 5.3** CT$_{\text{max}}$ of *N. lugens* acclimated at 15 (white bars), 23 (black bars) and 30°C (cross-hatch bars). Mean CT$_{\text{max}}$ of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter are not significantly different (p $\leq$ 0.05); n = 20 for each life cycle stage.

**Figure 5.4** Heat coma temperature (HCT) of *N. lugens* acclimated at 15 (white bars), 23 (black bars) and 30°C (cross-hatch bars. Mean HCT of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter are not significantly different (p $\leq$ 0.05); n = 20 for each life cycle stage.

**Figure 5.5** Lower lethal temperature (LLT$_{50}$) of first instar nymphs and adults of *N. lugens* acclimated to 15°C (white bars), 23°C (black bars) and 30°C (cross-hatch bars). Mean LLT$_{50}$ of A) first instar nymph and B) Adults. Mean values with the same letter are not significantly different (p $\leq$ 0.05); n = 50 at each exposure temperature.

**Figure 5.6** Upper lethal temperature (ULT$_{50}$) of first instar nymphs and adults of *N. lugens* acclimated to 15°C (white bars), 23°C (black bars) and 30°C (cross-hatch bars). Mean ULT$_{50}$ of A) first instar nymph and B) Adults. Mean values with the same letter are not significantly different (p $\leq$ 0.05); n = 50 at each exposure temperature.

**Figure 6.1** Range of development times for the nymphal stages of *N. lugens* after exposure at the ULT$_{50}$. N = 50 for control (31 male and 19 female) and treatment (21 male and 29 female) groups.
Figure 6.2 Range of development times for adults of N. lugens after exposure as first instar nymphs at the ULT\textsubscript{50}. N = 50 for control and treated groups (gender ratio as in Figure 6.1)………………………………………………………………………………………………121

Figure 6.3 Mean number of eggs per female after exposure of first instar nymphs and adults of N. lugens at their ULT\textsubscript{50}. N = 20 pairs for each mating combination. Mean values with the same letter are not significantly different at p < 0.05 level……………………………………………..................................123

Figure 6.4 Mean number of eggs per female after exposure of adults of N. lugens at their ULT\textsubscript{50}. N = 20 pairs for each mating combination. Mean values with the same letter are not significantly different at p < 0.05 level………………………………………………………………………………………………124

Figure 6.5 Range of egg development times after exposure of first instar nymphs and adults of N. lugens at their ULT\textsubscript{50}. N = 20 pairs for each mating combination……………………………………………………………125

Figure 7.1 Apparatus used for quantifying honeydew produced by N. lugens on rice plants over time……………………………………………………………………………………...............139

Figure 7.2 Honeydew production/12 h by nymphs of N. lugens feeding on rice plants for 15 days: A) control nymph males, B) control nymph females, C) treated nymph males and D) treated nymph females. N = 10 for each group………………………………………………………………………………………………141

Figure 7.3 Honeydew production/12 h by adult N. lugens feeding on rice plants for 21 days: A) control adult males, B) control adult females, C) treated adult males nymphs and D) treated adult females. N = 10 for each group………………………………………………………………………………………………144
List of Tables

Table 2.1 Mean (± SE) of arena surface and ambient temperatures in relation to the reference temperature at six constant temperatures in arena of different sizes……………………………………..38

Table 3.1 Mean (± SE) weight and sources of insect materials………………………………………..49

Table 5.1 Mean CT_{min} and chill coma temperature (CCT) ± SE of N. lugens acclimated to 15, 23 and 30°C (n= 20 for each life stage and sex)……………………………………………………………..90

Table 5.2 Mean CT_{max} and heat coma temperature (HCT) ± SE of N. lugens acclimated to 15, 23 and 30°C (n= 20 for each life stage and sex)……………………………………………………………..94

Table 5.3 LLT_{50} and ULT_{50} of nymphs and adults of N. lugens at 15°, 23° and 30°C…………98

Table 5.4 Temperature differential (°C) in thermal thresholds of N. lugens after acclimation at 15° and 30°C in comparison with a population maintained at 23°C…………………………………….102

Table 7.1 Mean honeydew production (mm²/12 h) by temperature-treated and control nymphs of N. lugens (n =10)…………………………………………………………………………………………………….142

Table 7.2 Mean honeydew production (mm²/12 h) by temperature-treated and control adults of N. lugens (n = 10)…………………………………………………………………………………………………….145
CHAPTER 1

General Introduction

Climate change related to regional warming is a critical issue that has been widely discussed and debated over recent decades (Rosenzweig et al., 2008). The global average warming near the Earth’s surface is predicted to lie in the range of 1.5° to 4.5°C over the next century (Houghton et al., 1990). However, this warming effect is not consistent across the globe, and both increases and decreases in temperature will occur in different regions. Furthermore, the wider effects of climate variability will be observed in terms of changes in patterns of rainfall, melting of glaciers and polar ice, and accompanying rises in sea level (Heong et al., 1995). Thus, climate change has the potential to be a major threat to species survival and ecosystem structure and function (Hulme, 2005), since different plant and animal species have different climatic requirements for growth, survival and reproduction that in turn, limit their geographic distribution, abundance and interactions with other species (Gutierrez et al., 2008).

Walther et al. (2002) show that the distribution of various organisms has already been altered as a result of changing environmental conditions. For example, some butterfly species, including non-native species from adjacent areas, appear able to track the trend of warming quickly and become part of the biota in new areas. The broad scale interactions between atmospheric composition, climate change and human, plant and animal health are thus of major importance and require urgent study to identify undesirable changes and achievable mitigations and solutions. Carrington (2011) reports that the estimated number of described species in the world are around 8.7x10^6 of which about 75% are thought to be insects. Thus, there is no doubt that one the most important
components of the world’s biodiversity are the Insecta. In addition, insects are not only the largest group with the greatest biological diversity, they also carry out several important functional roles in the biosphere, such as herbivory, decomposition, predation, parasitism and pollination (Herrera et al., 2002; Krimmel, 2012). In general, the species richness of insects in tropical areas is greater than in temperate areas (Larsen et al., 2011a). Similarly, diversity and ecosystem complexity of insects decrease with latitude, but increases in temperature are likely to modify these relationships (Wilf and Labandeira, 1999). The general prediction is that an increase in temperature would move distributions northwards and to higher elevations; also, temperature is likely to have significant and rapid impacts on distributions and abundance because of the ecophysiological features of insects: short life cycles, high mobility, reproductive potential, and physiological sensitivity to temperature.

This study will investigate the impact of climate change, principally higher temperatures, on a tropical insect of major agricultural importance. Among the abiotic factors that are changing through this phenomenon of ‘climate warming’ (temperature, CO₂, UVB, precipitation), temperature is likely to have the most direct affect on insects through the processes of development, reproduction and survival (Bale et al., 2002). Moreover, whilst many studies on the effects of climate warming have focused on polar and temperate species, there has been much less attention given to tropical insects, perhaps because it has been assumed that organisms that already experience high temperatures may be able to cope with even higher temperatures in the future. In fact, the reverse may actually be true, hence the need for ecophysiological studies on tropical species.
1.1 Climate change

Climate change is a natural process that has occurred since the beginning of the Earth’s evolution, about 4-5 billion years ago (Jansen et al., 2007). The climate system is based around interactions between physical, biological and chemical processes in the atmosphere, hydrosphere, biosphere and geosphere, and driven by energy from the sun in the form of solar radiation (Ingersoll, 1990). A proportion of the solar radiation reaching Earth’s surface is scattered or reflected by clouds, aerosols, dust and other particles, while a portion is absorbed and trapped as heat in the atmosphere, comprising water vapour, CO₂, CH₄, N₂O and O₃. This phenomenon is known as the natural greenhouse effect (Figure 1.1).

There are two important consequences of this natural greenhouse effect. Firstly, the planet’s surface temperature warms from around -18°C to about 15°C and without this warming effect there could be no life on Earth. Secondly, without this, night-time temperatures would be much lower than they are (King, 2005). At present time, however, many climatologists predict a significant increase in temperature above that associated with long term natural process, attributable to the increasing concentration of atmospheric ‘greenhouse gases’. The Intergovernmental Panel on Climate Change (IPCC) reported that natural ecosystems are being affected by global warming with regard to a gradual but accelerating increase of atmospheric greenhouse gases (Lobell et al., 2008; Knutson et al., 2010). The global increases in CO₂ concentration are due primarily to fossil fuel use and land-use change, while those of CH₄ and N₂O are mainly due to agriculture. Consequently, the mean Earth surface temperature has increased by about 0.3-0.6°C since the late 19th century (Alley et al., 2007).
Temperature is not only the abiotic factor that will change as a result of ‘global warming’. There will be changes in precipitation, UVB penetration, creating some ‘extreme situations’, such as flooding, storminess and drought (Lal et al., 2001; Albritton et al., 2002), and there is evidence for some of these effects in different parts of the world over the most recent 10 years. It is clearly a very challenging problem for the world and its people to live with, or overcome the consequences of global climate change. There are international recommendations to reduce greenhouse gas emissions to the 1990 level; but this is difficult to achieve because of the reluctance of rapidly developing economies to constrain their industrial production. It is also a high priority to carry out research on the impact of climate change on the vulnerability of ecosystems because of the threat posed to the survival of many species (King, 2005).

Figure 1.1 Components and interactions of the global climate system (from Ahlonsou et al., 2001).
Many climatologists use models to predict the impacts of climate change, focusing attention on a number of key factors and processes in both aquatic and terrestrial ecosystems. It has been concluded that, climate change and climate ‘extremes’ will affect all levels of life, from individuals, through populations to ecosystems and the eco-region level. Xiangdong et al. (2007) report that changes in the global climate have become more interconnected, having both direct and indirect effects on ecosystem responses. The severity of impacts of climate change on ecosystems will vary both spatially and temporally.

A distinction can be made between changes in climate as represented by meteorological data and the observable environmental consequences. Thus climate data and statistics can identify abnormal extremes such as very low or very high daily temperature, or very heavy daily or monthly rainfall in relation to ‘normal patterns’. The environmental consequences are more complex because they are part of a natural cyclical of events in which accelerated changes in the rate may be indicative of a link to recent climate warming (Easterling et al., 2000). For example, Member and Barrie (2008) conclude that extreme weather events such as heat waves, droughts, floods and hurricanes are now occurring more frequently and with greater intensity, as would be predicted through the effects of global warming. For instance, hurricanes at category 4 and 5 have increased 75% since 1970. Mountain glaciers are thinning, while snow cover is retreating earlier in the spring. In addition, permafrost is melting and sea ice in the Arctic is shrinking faster than expected and has thinned by up to 40% in recent decades (King, 2004). Tandong et al. (2004) estimate that the high Asia glaciers in China are retreating rapidly under global warming and will have disappeared by the end of this century. Scholze et al. (2006) have carried out a risk analysis for the impacts of climate change on various world ecosystems using 16 climate models,
concluding that forest loss in Eurasia, eastern China, Canada, Central America and Amazonia are a high risk, but with forest expansion into the Arctic and savannas. Further, it also reported that extreme temperatures -high and low- are likely to increase in this region (Alley et al., 2007).

At the population level, Miles (1994) reported that climate change can affect populations in one of three ways: changes in abundance and distribution, rapid adaptation to changing environmental conditions, or extinction, at least locally. Recently, it was predicted that 15 – 37% of current species may be extinct by 2050 (Lewis, 2006). There is, however, a counter view that terrestrial animals may not be affected by temperature elevation and concomitant changes in vegetation based on studies of fossil insects - Coope (1986) suggests that animals were able to respond to previous climate fluctuations - thus, individual species adjust to changing conditions of their geographic environment rather than by evolutionary change in morphology. Miles (1994), however, points out that the expected pattern of change is unique both in terms of the accelerated rate of predicted global temperatures and the fragmented nature of natural habitats.

Global climate change is also a major concern in many areas of the world because of likely effects on the socio-economy, farming and politics (Lepetz et al., 2009). Jung et al. (2009) reported that South-East Asia is one of the most ‘at risk’ regions of the world, which could critically obstruct the region’s sustainable development and poverty alleviation policies if the problem was not effectively tackled. Between 1951 and 2000 the mean temperature increased by 0.1-0.3°C, there was a downward trend in precipitation, whilst sea levels rose by 1 - 3 mm per year (UNFCCC, 2007). The area is geographically vulnerable as the tropical climate may suffer from a more severe impact of rising sea level with approximately 563 million people living along coastlines with fast growing populations. Simultaneously, most Asian livelihoods rely heavily on
agriculture and food security, both of which are vulnerable to natural habitat change and an increasing intensity and frequency of climate-related disasters such as droughts, heat waves, floods, landslides, fires and storms. It is not only the heavy reliance on the agricultural sector, but also, the deep dependence on natural resources, especially forestry, which have been over-exploited in recent years. At present, agro-ecosystems are facing challenges from global warming and this issue has become a key concern since global food production resources are already under pressure from a rapidly increasing population (Tao et al., 2008).

Food security is defined by the Food and Agriculture Organization (FAO) as the situation in which all people have physical, social, and economic access to sufficient, safe, and nutritious food without interruption (Schmidhuber and Tubiello, 2007). The global agricultural sector plays an essential role in providing food, thus aiding human survival and societal development, so that people can sustain their own livelihood. Crosson and Anderson (1994) reported that the increased demand for food linked to population growth will double from the late 1980s to 2030 on a global scale. Rice is the dominant crop in Asia and over 90% of the global total rice production is grown in the area (Wu, 2010). Granamanickam (2009) indicated that rice is a crop of world-wide importance for both export and ‘home consumption’ for many countries which collectively represent over 50% of the world’s population. The paddy rice field ecosystem is therefore one of the largest managed ecosystems on Earth. However, such agro-ecosystems are sensitive to both short-term and long-term changes in climate. Climate change may affect agriculture in three main ways (Parry, 1990). Firstly, increased atmospheric CO$_2$ concentrations can have direct effects on the growth rates of crop plants and weeds. Secondly, the levels of temperature, precipitation, and sunshine may be altered by changes in CO$_2$ concentration. Thirdly, rises in sea level may lead to
the loss of farmland by inundation and to increasing salinity of ground water in coastal areas. This is a critical problem of great importance, especially in tropical regions, where there are high ambient temperatures throughout the year (Matthews et al., 1995). Long term changes in climate and weather patterns can have a major influence on agro-ecosystems, especially on the occurrence and prevalence of insect pests and diseases (Chakraborty et al., 2000). Under different climatic situations insect pests cause a significant loss to world food production. Temperature patterns, rainfall or humidity influence insect development and distribution, and many studies have shown that the rate of insect development increases with temperature up to an optimum, though this optimum varies between species (Khan et al., 2009). The eco-physiology of tropical insects living in agricultural environments has however, not been well studied, and this is particularly true in terms of the impact of a changing climate.

In summary, to be sufficiently prepared for the effects of global warming, it is important to predict the impact of global warming on both natural and agricultural ecosystems. There is increasing scientific evidence to show that climate change influences both flora and fauna of all types of ecosystems from the tropics to the poles, and from the species to the community level (Walther et al., 2002). Recent climate warming has also modified species distributions through temperature-related range shifts (Parmesan et al., 1999; Thomas et al., 2001). Thus, management policies are urgently required to maintain the stability and health of ecosystems, and more specifically with regard to the focus of this project, to understand the likely effects of higher temperatures on insect herbivores in tropical climates.
1.2 Impacts of climate change on insects

Insects will respond to climate change in various ways, but two responses have already been detected over recent decades. Firstly, changes in phenology i.e. the timing of annually recurring events (emergence from overwintering, timing of oviposition), and secondly, changes in geographical distribution, such as northerly or altitudinal range expansion (Parmesan, 2007). There have been many studies on phenological events in insects. Different species inhabit a diverse range of locations with varying temperatures, both predominantly high (tropics) or low (polar areas), and this has a major effect on phenology.

Phenological events such as the timing of bud burst, egg hatch and migrations are sensitive to climate and have been used as bioindicators of responses to climate warming (Bale et al., 2002; Boudon-Padieu and Maixner, 2007). In addition, Hodkinson (1997) reported that the availability, phenology, and quality of host plants will be influenced by a changing climate. The effects of climate change will therefore impact on insect herbivores directly (development, reproduction and survival), but also indirectly through changes in food quality with the potential to alter both the nature and strength of many plant-herbivore interactions. Indeed, some effects of global warming on insect populations are already apparent. According to one survey, about 940 species from a total of 1,600 are showing some effects of climate change (Musolin, 2007). Karban and Strauss (2004) report that the range of the meadow spittlebug, *Philaenus spumarius*, has moved progressively northwards along the California coast since 1988, with temperature and humidity the chief factors affecting survival and reproduction.
Insect distributions will also be affected by climate change; their adaptability and genetic variability will in some cases enable them to exploit new environments (Goudriaan and Zadoks, 1995). It is important to be able to predict potential distributions, especially of pest species (both non-indigenous and indigenous), to determine the likely impacts of global climate change on natural and agro-ecosystems (Baker et al., 2000). In general, insect diversity and ecosystem complexity decrease with latitude, but increases in temperature may modify these relationships. The key prediction is that an increase in temperature would move distributions both northwards in latitude and upwards in elevation (Parmesan, 1996).

Temperature can have a significant and rapid impact on distributions and abundance because the main eco-physiological trait of insects (e.g. life cycle duration, mobility, reproduction), are all sensitive to the thermal environment. Insect distributions can be altered by climate change in several ways, both direct and indirect. Direct effects could involve impacts on insect development and survival, changes in host defence physiologies, while indirect effects would include changes in natural enemy and competitor abundance (Ayres and Lombardero, 2000). For example, Gutierrez et al. (2008) studied the effect of climate change on poikilotherm tritrophic interactions and found that the cold intolerant pink bollworm (a pest of cotton) expanded its range and migrated to formerly inhospitable areas in the San Joaquin Valley, California where heavy frosts had previously prevented survival. Research on the effects of temperature and climate change on insects has however, focused primarily on changes in summer temperatures of 1-2°C on processes such development, reproduction, abundance and distributions (Strathdee and Bale, 1993), and mainly in polar and temperate climates (Parmesan et al., 1999; Bale et al., 2000; 2001). There has been less focus on the impacts of higher winter temperatures on survival, and
even less attention on the possible effects of high temperatures on insects, especially those living in tropical areas.

Climate change is a global phenomenon, but the extent of warming will vary between different climatic zones. For example, it is envisaged that increases in temperature will be greater in polar areas and the temperate zone than in the tropics – this may explain why many field studies have been carried out in these areas. Also, there may have been an assumption in the research community that because the extent of climate change in the tropics may be less than elsewhere, and tropical insects can tolerate high temperatures, they would not be as much affected by increases in temperature as other species. There is now increasing recognition that some tropical insects may be living close to their upper thermal limits, and even relatively small increases in temperature may become lethal or sub-lethal for such species (Talekar and Shelton, 1993; Krebs and Loeschcke, 1996; Krebs and Feder, 1998; Klok et al., 2004; Nice and Fordyce, 2006; Lapointe et al., 2007).

1.3 Thermal biology and insect physiology

Environmental factors such as temperature, humidity and oxygen availability have considerable influence on ectothermic animals (Huang and Tu, 2008). Nespolo et al. (2003) indicate that among these abiotic factors, temperature has the most profound effect because physiological functions and behavioural performance are influenced by the animal’s body temperature. The thermal biology of insects can be described by two main hypotheses.
Firstly, temperature is one of the most important factors affecting biological processes in insects. Secondly, the direction and rate of biochemical processes that underlie insect performance can be described by the laws of thermodynamics (Angilletta Jr. et al., 2006). All levels of biological organization involve adaptive responses to thermal heterogeneity, from the expression of genes to the behaviour of organisms but these responses happen on different scales.

Insects are the only invertebrates with wings and have lived on the earth for 350 million years; they have evolved their life histories in response to changing environmental conditions (Triplehorn and Johnson, 2005). Insects have a limited ability to regulate their body temperature; for this reason, selection of suitable thermal microhabitats is an important factor in the maintenance of optimal body temperature. At high temperature, insects are highly vulnerable to injury because body temperature in such small poikilotherms can increase rapidly to lethal levels from solar radiation or artificial heat. Maintenance of appropriate water balance is also a challenge at high temperature (Turnock, 1999).

1.3.1 Background

Insects are poikilotherms or cold-blood animals; this means that their body temperature ($T_b$) is essentially the same as the environmental temperature and that key processes and behaviours such as development, reproduction and activity are all dependent on temperature (Speight et al., 1999). Different species can live in markedly different environments, from the tropics to the poles, but for many species, information on their favourable range and optimal temperatures for various processes is lacking.
The generalised relationship between body temperature and performance is shown in Figure 1.2. The graph is representative of most quantitative traits such as egg production, developmental rate or other metabolic processes that have critical thermal limits. These limits are at the low and high extremes and are termed the critical thermal minimum (CT$_{\text{min}}$) and the critical thermal maximum (CT$_{\text{max}}$), respectively, whilst, the range within which the insect can live and express its physiological or behavioural performance is called the tolerance zone (Huey and Bennett, 1990). Physiological performance increases progressively with body temperature up to a maximal value at the optimum body temperature ($T_o$), and then decreases rapidly above this temperature (Turnock, 1999). The ability of insects to cope with thermal stress is achieved in one of two main ways - thermoregulation of body temperature through behavioural adaptation and physiological and biochemical mechanism - and changes in thermal sensitivity, including both short-term processes, such as acclimatization and long-term processes, such as evolutionary adaptation (Huey and Stevenson, 1979).

**Figure 1.2** Hypothetical performance curve of an insect as a function of body temperature, showing the 80% performance breadth ($B_{80}$), CT$_{\text{min}}$ and CT$_{\text{max}}$ (from Chown and Nicolson 2004).
1.3.2 Thermoregulation in insects

Angilletta Jr. et al. (2006) define the process of thermoregulation as the maintenance of the mean or variance of body temperature ($T_b$) maintenance in relation to the mean or variance of the environmental temperature ($T_e$) by behavioural, physiological or morphological strategies. Thus, regulation and homeostasis are the principle paradigms of comparative and ecological physiology (Hertz et al., 1993). An insect’s $T_b$ rises and falls with the $T_e$ but this response can be altered when the range of $T_e$ is greater than the range within which the organism is generally active. However, insects need mechanisms by which to survive under conditions of thermal stress because of their limited ability to regulate $T_b$ (Bale and Hayward, 2010) and this is achieved by processes collectively known as capacity adaptation (Chown and Nicolson, 2004). Potential injury is avoided or minimized by a combination of behavioural, physiological and biochemical responses that enable some insects to sustain a stable temperature while exposed to a broader range of $T_e$ (Triplehorn and Johnson, 2005).

Insects use behavioural adaptation as a first response to high temperature stress such as habitat selection, basking intensity, restriction of activity periods and selective exploitation of environmental thermal fluxes (Yang et al., 2008). Many insects effectively regulate $T_b$ by using behavioural adjustment that maximize temperature-dependent growth rates which therefore, often affect their fitness (Nice and Fordyce, 2006). For example, a fly can avoid a ‘hot area’ under extreme sunshine by flying to a shaded location. Likewise, the simple movement of a caterpillar from the upper to lower surface of a leaf can decrease the body temperature by several degrees celsius in just a few minutes (Turnock, 1999). Conversely, in winter, honey bees survive in their hive with some activity while most other insects go into a more dormant state. Triplehorn and
Johnson (2005) show that the bees form a cluster in the hive and activity of their thoracic muscles raises the hive temperature to a much higher level (34° to 36°C) than the T_e (14°C). Forsman (2000) also reports that sex, reproductive condition, feeding status, disease, and time of season are all factors that may cause individuals to select different T_b.

The second approach to the prevention of injury caused by thermal stress involves physiological and biochemical mechanisms. To raise their T_b, insects can bask in sunshine or use an active process such as shivering. For example, the T_b of butterflies and grasshoppers in flight may be 5° or 10°C above the T_e. Also, moths and bumble bees which have hair and scales as insulation can have flight muscle temperatures 20° or 30°C above the T_e because of increasing metabolic rate during flight. Heath et al. (1971) describe the shivering mechanism in which the muscles that are normally antagonists in flight contract simultaneously. Each antagonist provides the loading of the isometric contraction of its opponent. When the thorax warms, each muscle is fired more frequently and each contract is more vigorous. Consequently, the T_b rises linearly and the insect’s metabolism also increases as indicated by O_2 consumption. In contrast, some insects use evaporative cooling or sweating to lower their T_b. The cooling of a small insect is moderately fast since its T_b in flight is very close to T_e (Triplehorn and Johnson, 2005). For biochemical adaptation or at the cellular level, insects improve their chances of survival by the synthesis of stress proteins and other key metabolites (Turnock, 1999).

Figure 1.3 summarises the mechanisms used by insects to regulate their body temperature or control the exchange of heat with their environment. These mechanisms are largely dependent on the absorption heat from the environment, hence environmental conditions determine whether heat is gained or lost. Briefly, solar radiation, either direct or via the heating of rocks or soil, is
the most important source of heat, whilst heat loss is mainly through the processes of conduction, convection or radiation from its body (Wharton, 2002). Conduction is the direct transfer of heat when objects of different temperatures come into contact, whilst heat is lost by convection when the air temperature is below the insect $T_b$. Insects exchange radiation with each other and with the sky, whereby warmer objects lose heat to cooler objects (Purves et al., 2001).

Figure 1.3 Mechanisms of heat gain and loss in insects (adapted from Wharton, 2002).
In general, these processes can be described using the principles of thermal equilibrium or the zeroth law of thermodynamics. This law is important as an aid to understanding the concept of body temperature regulation in insects. Bakken (1992) provides a summary that insects can adjust their $T_b$ to approach the same $T_e$ by heat transfer and the zeroth law of thermodynamics is used to explain this thermal equilibrium process.

The zeroth law states that if there are two thermodynamic systems, $X$ and $Y$, which are in thermal equilibrium with another body $Z$, then the bodies $X$ and $Y$ will also be in thermal equilibrium with each other. The zeroth law can explain the principles of thermoregulation in insects as shown in Figure 1.4. When an insect (body $Y$) is placed in a container (body $X$), the temperature of two systems (bodies $Y$ and $X$) can be compared by a thermometer (analogous to body $Z$). The thermometer is allowed to come into thermal equilibrium with body $X$ at the beginning of the measurements. After that the thermometer is allowed to come into thermal equilibrium with body $Y$ by placing it in contact with $X$ and $Y$ in turn. The thermometer will show the comparative temperatures of the two bodies $X$ and $Y$. The energy exchange between $Y$ and $Z$ or between $X$ and $Z$ is insignificant because the thermometer or body $Z$ is small compared with $Y$ and $X$. Further, during temperature measurement there is no change in the individual energies of body $X$ and $Y$. Thus, two objects, bodies $Y$ and $X$, when in thermal equilibrium, will have the same temperature.
Figure 1.4 The zeroth law of thermodynamics; two objects (Y and X) that are independently in thermal equilibrium with a third object (Z), are also in thermal equilibrium with each other.

In conclusion, insects are viable under a wide range of $T_b$, and their physiological processes and behavioural activities are usually maximized at moderate to relatively high $T_b$ (Yang et al., 2008). However, the extent of thermoregulation varies among species and in different environments. Some species have a broad range of $T_b$ (termed thermoconformers), while others are more precise ‘thermoregulators’ that are active under a narrower range of $T_b$ (Row and Blouin-Demers, 2006). Bowler and Terblanche (2008) report however, that there is still a lack of knowledge on the thermal biology in insects in areas such as temperature tolerance and thermoregulation strategies, processes that are increasingly important in an era of climate change.
1.3.3 Temperature tolerance

There are several ways to measure the physiological responses of insects to temperature extremes (Bowler, 2005). The most commonly reported variation in thermal tolerance is that associated with different life stages (Krebs and Loeschcke, 1996). This study will focus on three indices of high temperature tolerance - the critical thermal maximum (CT$_{\text{max}}$), heat coma temperature (HCT) and the upper lethal temperatures (ULT) and three indices of low temperature tolerance - the critical thermal minimum (CT$_{\text{min}}$), chill coma temperature (CCT) and the lower lethal temperatures (LLT) in two life cycle stages – a juvenile stage (nymph or larva depending on the species) and the adult. In general, insect responses are highest in the intermediate $T_b$ range and they reduce responses at higher or lower body temperature. To characterize thermal sensitivity in insects, the lethal temperature is one of three descriptive measurements. The descriptive measurements are composed of the optimal temperature range, the thermal performance breadth, and the tolerance range which include the ULT and LLT (Huey and Stevenson, 1979).

1.3.3.1 Critical temperature (CT) and coma temperature (CT)

Changes in temperature have profound effects on biological processes (Wharton, 2002). In general, insect responses are highest in the intermediate $T_b$ range and are reduced at higher or lower body temperature, as summarised in Figure 1.5. As temperature increases or decreases from the optimum and becomes more extreme at the both end of the scale, the continuation of such change results first in the disruption of movement, as locomotor activity becomes disorganised and the insect loses the ability to move in a coordinated way; in ecological terms,
this would prevent it from escaping from the conditions that will promptly lead to its death (Hanna and Cobb, 2007).

The low and high temperatures at which these responses occur are termed the critical thermal minimum (CT$_{\text{min}}$) and the critical thermal maximum (CT$_{\text{max}}$). Then, close to the limit of the tolerable range of temperatures, the insects will enter a cold or heat coma state at which the last ‘twitch’ of an appendage (leg or antenna) occurs (Hazell et al., 2010a). These temperatures are described as the chill coma temperature (CCT) and heat coma temperature (HCT). Once the temperature limits are exceeded, the insects will die (Wharton, 2002; Chown and Nicolson, 2004). The distinction between the respective critical thermal temperatures and the coma temperatures can usually only be distinguished in systems that video record the movements of the insects.

1.3.3.2 Lethal temperature (LLT and ULT)

At temperatures below and above the optimum, metabolism slows and eventually ceases due to the damaging and lethal effects of low and high temperature. Thus all species will have a lower (LLT) and upper (ULT) lethal temperature. The ULT will normally be higher than the CT$_{\text{max}}$ and heat coma temperature. However, the difference between the heat coma and ULT is sometimes small e.g. around or less than 1°C, and insects experiencing heat coma may be unable to recover, so the heat coma is effectively the ULT (Hazell et al., 2010a). There are a range of methods used to measure the ULT including ‘direct plunge’ where insects are transferred directly to a potentially high temperature, and ‘dynamic methods’ where the temperature is increased gradually and mortality is assessed at a sequence of increasingly higher temperatures. The crucial
factor is that the ULT is expressed as a temperature in which mortality occurs after a very brief exposure (seconds or a few minutes), though death may occur post-exposure, hence estimates of mortality are usually made some days after experiencing the thermal stress (Hazell et al., 2008).

Briefly, for determining the ULT, insects are heated at a set rate (e.g. 0.5°C min$^{-1}$) from the rearing temperature (e.g. 20°C) to a series of pre-determined temperatures. When the temperature in the alcohol bath increases up to the target temperature, all samples are held at that temperature for a short period of time sufficient to ensure that all individuals in the sample experience the set temperature, after which the sample is returned to the rearing temperature at the same rate as used for warming (Huey et al., 1992). Most studies expose insects directly to the assay temperature with equilibration taking place in less than a minute (Chown and Nicolson, 2004), but the time required for equilibration is likely to vary depending on the size of the specimen (and therefore the sample) and the rate at which the insects are warmed, and these factors need to be considered in the experimental design.

To conclude this section, there is no doubt that the biodiversity of insects in tropical areas is greater than other regions, but there have been relatively few studies on the temperature tolerance of such species, hence there is little information on which to develop an understanding of how they will respond to, or be affected by, climate warming. Bale et al. (2002) indicate that many studies on the effects of climate change impacts on insects have focused on a limited range of taxa e.g. butterflies, with few reports on species that are important as agricultural pests (Boudon-Padieu and Maixner, 2007). For these reasons, this project focuses on a tropical insect, the brown planthopper *Nilaparvata lugens* (Stål), which is a pest of one of the world’s major crops, rice.
Figure 1.5 Responses to temperature in a hypothetical organism (adapted from Wharton, 2002; Chown and Nicolson, 2004).

1.4 Ecology and biology of the brown planthopper, *Nilaparvata lugens*

*Nilaparvata lugens* (Stål) (Homiptera, Delphacidae) is commonly known as the brown planthopper and is one of the most devastating pests of rice throughout Asia and causes serious yield losses in many countries (Cuong et al., 1997; Senthil-Nathan et al., 2009). Before the green revolution in Asia, *N. lugens* was regarded as only a minor pest; however, since the 1940s there have been massive outbreaks caused by a combination of insecticide resistance and an expansion in rice cultivation (Nagata et al., 2002). The control of *N. lugens* is based around the use of chemical insecticides and rice cultivars with resistance to brown planthopper. These methods are however, not effective as the pest has developed new biotypes (Suzuki et al., 2006).
International Rice Research Institute (IRRI) (1976) categorize *N. lugens* into to three biotypes based on their ability to feed on rice with different resistance genes. Biotype 1 are populations of *N. lugens* that cannot infest any rice variety with the resistance genes *Bph 1* and *Bph 2*. Strains of the pest that can feed on rice varieties with the resistance genes *Bph 1* and *Bph 2* are defined as biotype 2 and biotype 3 respectively. There is also an evidence that *N. lugens* can shift from a simple to more complicated biotype. For example, although commercial rice varieties have carried the resistance genes since the 1970s, biotypes of *N. lugens* have evolved that are able to feed on these ‘resistant’ varieties of rice (Tanaka, 1999).

1.4.1 Development and life cycle

The mechanism of chromosomal sex determination in *N. lugens* is of the XO type (Saitoh et al., 1970). The female has 16 chromosomes and carries identical sex chromosomes (XX). Thus, the female produces only one type of ovum. The male has 15 chromosomes since it has only one sex chromosome and is represented as XO. If the X-carrying ovum is fertilised by an X-carrying sperm, the resulting zygote XX will develop into a female. On the other hand, if there is no sex chromosome carried on the sperm that combines with an X-carrying ovum, the zygote XO will develop into a male offspring. Ichikawa and Ishii (1974) reported that males cannot copulate within the first 24 h after emergence, but then copulation ability increases over the next 4-5 days after emergence and then decreases. Adult males on rice plants are attracted by the abdominal vibration of females over a distance of up to 80 cm. Takeda (1974) reported that a single male could copulate with up to nine females over a 24 h period, whilst a female could mate two or more times during her life time. After mating, females lay eggs by penetrating plant tissue with
the ovipositor where eggs are laid in groups (Hattori and Sogawa, 2002), mainly in leaf sheaths, but also in the leaf blade.

The life cycle of *N. lugens* is an example of incomplete metamorphosis or hemimetabolism i.e. egg, nymphs and adult. The nymphaial stages take between 7 to 15 days to complete development under tropical climatic conditions (Mochida and Okada, 1979). Nymphs have a similar appearance to adults but are smaller, have different coloration, and no functional wings. Wing buds appear during development, and are clearly visible in the fifth instar. Nymphs and adults have a rostrum for ‘sucking’ sap, and all stages can ‘hop’ if disturbed. The nymphaial instars can be individually discriminated by the appearance of the mesonotum and metanotum of the thorax, and by colour and body size.

Adults of *N. lugens* have two wing-forms with long (macropterous) or short (brachypterous) wings (Ge et al., 2011). The macropterous forms are potential migrants and colonize new areas, sometimes long distances from the site of origin (Yu et al., 2001). After settling on the rice plants, the insects lay eggs; the next generation of adults are mostly brachypterous. The short winged adult morph lays more eggs than macropterous adults (Dyck et al., 1979). As the crop matures, there is a switch to macropterous adults as the dominant adult morph, which disperse to other areas.

1.4.2 Feeding behaviour

*Nilaparvata lugens* is a phloem-feeding insect on rice plants. They have elongate mouthparts for piercing and sucking fluids in the phloem and xylem tissue (Dupo and Barrion, 2009; Seo et al., 2009a). When *N. lugens* feeds on rice, it pierces the phloem and ‘sucks out’ the nutritive liquids
that form its diet. This feeding activity results in the deposition of salivary sheaths at the feeding site (Zhang et al., 2004; Senthil-Nathan et al., 2009). Tjallingii (1978) developed an electrical penetration graph (EPG) technique to monitor and record homopteran feeding behaviour quantitatively. This method has been used to investigate the correlation between EPG waveforms and feeding behaviour in *N. lugens* by recording EPG and simultaneously observing honeydew excretion. El and Goodman (1993) reported that there are three distinct patterns of feeding behaviour of *N. lugens* in rice as represented by different EPG waveforms. Two of these patterns relate to feeding activity in the phloem and xylem tissue, whilst the third represents a complex of feeding activities such as salivation and stylet penetration into non-vascular tissues of rice plants.

Seo et al. (2009b) studied the relationship between the feeding behaviour of *N. lugens* on different rice varieties and survivorship. It was found that *N. lugens* could survive well on resistant cultivars carrying the *bph 1* and *bph 2* genes, even though they could not easily ingest phloem sap from such varieties. This observation indicated that limited phloem feeding ability on resistant rice varieties is not by itself an explanation for the observed mortality in *N. lugens* when feeding on certain cultivars, and that other factors, such as the ecological and physiological costs of overcoming resistance mechanisms in the plant may have a negative impact on survival.

1.4.3 Migratory behaviour and distribution

*Nilaparvata lugens* is a significant pest of rice that is widely distributed in Asia across tropical, subtropical and temperate regions. The main ecological features of *N. lugens* that contribute to this pest status are the high migratory ability and high fecundity (Kisimoto, 1979). The distribution of *N. lugens* in Asia where occasional outbreaks cause serious damage to cultivated
rice is shown in Figure 1.6. The dotted line in Figure 1.6 shows the area from which resistant germplasm originates (India and Sri Lanka). Khush (1979) reported that in East Asia, South-east Asia, and the Pacific Islands there was only biotype 1 of *N. lugens* before the resistant varieties of rice were introduced after which different biotypes evolved that were able to feed on rice varieties with *Bph 1* and *Bph2* resistant genes. The two types of winged adults of *N. lugens* appear to have different roles in the colonisation of new locations (Kisimoto, 1979). Long distance migrations are carried out by the long-winged form, while the short-winged form builds up the population in newly colonised areas. In addition, Kuno (1979) indicates that the initial distribution in newly colonised fields is random, but then becomes more patchy or aggregated over time as the short-winged adults have limited ability to travel.

**Figure 1.6** Distribution of *N. lugens* in Asia (Khush, 1979).
Nilaparvata lugens is known to make wind-assisted migratory flights each year to colonize the summer rice growing areas of China, Japan and South Korea (Rosenberg and Magor, 1983). It has been assumed that during the rice growing season in Korea, N. lugens migrate annually, mainly around mid-June to late July from the south-east part of China, carried by the south-westerly airflow and on the route of the depressions in the rainy season (Seo et al., 2009b). In the tropics, the migration of long–winged adults also occurs from mature crops to younger plants during harvest. An important factor that limits the distribution of N. lugens is a lack of overwintering ability (Dyck and Thomas, 1979). Thus, although N. lugens is widely distributed in Asia, its ecology differs between tropical and temperate areas. The main difference is that whereas populations in tropical areas can remain in paddy fields throughout the year, in temperate regions, there is an annual replacement by immigrants from southern regions because of the inability to survive through winter (Kuno, 1979).

Win et al. (2011) investigated the population fluctuations of N. lugens in Myanmar (a tropical sub-region) between the rainy and summer season to determine the influence of ecological factors such as relative humidity, temperature and rainfall on population abundance. The study of Win et al. (2011) found that N. lugens populations were highest between 64 and 74 days after transplanting (by mid September) associated with heavy rainfall, high temperature and high humidity, and were lowest in mid October, suggesting that low rainfall and low humidity were at least partially responsible for the decrease in population size. There may however, not be a direct effect between rainfall and population fluctuations of N. lugens, but via changes to the physiology and water relations of rice plants. Fluctuations in planthopper numbers were correlated with rainfall patterns during the first cropping season, but more with temperature and
relative humidity in the second cropping season. Thus temperature, rainfall and relative humidity can all influence planthopper populations, but these effects may differ during the two rice growing seasons.

1.4.4 Outbreaks and economic impacts

Nilaparvata lugens is an economically important pest that feeds directly on rice plants and also acts as a vector of viruses of rice, resulting in significant damage and yield losses (Dupo and Barrion, 2009). There were numerous N. lugens outbreaks in South-east Asia in the 1980s with densities as high as 1,000–2,000 per ‘hill’ (Kiritani, 1979). In rice cultivation a hill comprises 3-4 rice seedlings planted very closely together. It is believed that excessive use of urea as a nitrogenous fertilizer is one of the main causes of outbreaks as it increases the fecundity of N. lugens. The injuries caused by N. lugens to rice plants include a decrease in leaf area, photosynthetic rate, plant height, leaf and stem nitrogen concentration, chlorophyll content and dry weight. Heavy injury usually results in wilting, stunting, and finally death of the plant. This type of damage is called ‘hopper burn’. Nilaparvata lugens may also transmit the grassy stunt disease which can further reduce yield. The series of slits produced by females when depositing their eggs may also contribute to plant dieback (Zhang et al., 2004). Dyck and Thomas (1979) reported that annual yield losses caused by N. lugens and the grassy stunt disease in most of the countries where N. lugens are found has a value of more than 300 million US$. According to available data, the most extensive losses from N. lugens have occurred in Japan, Indonesia, Taiwan, Philippines, and India with estimates of annual yield losses amounting to 100, 100 , 50 , 26 and 20 million US$ respectively. It is evident that a greater knowledge of the ecophysiology
of *N. lugens* in an era of climate change will provide valuable information on future population trends of this serious pest of rice.

### 1.5 Aims

Against this background, the main aims of this project are to investigate the effects of higher temperatures as might be experienced through climate warming on the survival, mobility, feeding behaviour and acclimation ability of the brown planthopper *Nilaparvata lugens*. 
2.1 Abstract

Insect activity thresholds such as the $CT_{\text{min}}$ and $CT_{\text{max}}$ are important in understanding ecophysiological responses, including interactions between species, such as predator-prey relationships. The video-capture recording of insect behaviour within a cooled or heated aluminium block is a commonly used method for such investigations, but as with all similar techniques, has the problem that it is not possible to measure directly the temperature of the observed specimen without influencing mobility. In the aluminium block system, cooled or heated fluid circulates from a programmable alcohol bath within channels in the block. Calibration experiments were carried out to compare the reference temperature within the aluminium block with the surface and air temperatures in ‘arenas’ of different dimensions milled within the blocks, comprising the areas over which the insects moved during the video recordings. Arenas of three sizes were created with diameters and depth of 1.6 and 0.4, 2.5 and 0.8, and 4 and 0.8 cm respectively, in correspondingly larger blocks, to accommodate species of different sizes. With each block the surface and air (ambient) temperatures within the ‘observed arena’ differed from the reference temperature measured within the body of the block, attributable to a gain (low set temperature) or loss (high set temperature) of heat to and from the arena and the surrounding environment. When the small, medium and large arenas were cooled or heated to 10, 15, 20, 30, 40, and 50°C, the difference in arena surface and air temperatures
from the reference temperature was 2.4-3.5, 1.3-2.2, 0.6-1.1, 1.5-2.0, 2.7-6.6 and 4.4-7.7°C respectively. The lowest level of variation was observed when the set reference temperature was similar to room temperature (20°C). The data were used to generate calibration equations that were then used in experiments to determine activity thresholds such as the heat coma temperature. The importance of carrying out calibration experiments in studies of insect thermal biology is discussed.

2.2 Introduction

The study of insect ecophysiology aids the understanding of the role of the environment in shaping the diversity of physiological, morphological and behavioural features of insects (Feder, 1987; Bennett and Huey, 1990; Crill et al., 1996; Parmesan et al., 2000; Robertson, 2004; Kingsolver et al., 2007; O’Neill and Rolston, 2007; Klose et al., 2008; Overgaard et al., 2008; Huey et al., 2009; Hazell et al., 2010a). Many of the processes that occur in living organisms depend on, or are strongly influenced by temperature, hence, the accurate determination of temperature is an important physiological measurement (Bursell, 1964; Bakken, 1992; Addo-Bediako et al., 2000; Berrigan, 2000; Bale et al., 2002; Both et al., 2005; Angilletta Jr. et al., 2010).

Experiments on the thermal biology of insects take two main forms: those in which the insects are constrained or immobile such that the temperature of an individual or sample can be measured by direct body contact (e.g. lethal temperatures), and those in which the specimens need to be able to move freely for observations to be made (e.g. determination of activity...
thresholds). The latter type of experiment presents a number of challenges and is also subject to error. For example, the environment in which an insect is exposed must be cooled or heated e.g. via fluid from a circulating alcohol bath, but the temperature of the source may differ from that achieved in the ‘exposure environment’. Also, insects may take a period of time to reach the required exposure temperature. This chapter describes a series of preliminary experiments to determine the ‘exposure parameters’ to be used in the studies with *N. lugens*.

The system used to assess the activity thresholds of *N. lugens* involves the circulation of a cooled or heated fluid within an aluminium block with the video-recording of insect activity within an ‘arena’ milled out the block. A temperature sensor placed within the block provides a simultaneous temperature reading that can be ‘cross-related’ to the observed behaviour of the insects (Cokendolpher and Phillips, 1990; Huey et al., 1992; Bauwens et al., 1995; Crill et al., 1996; Klok et al., 2004; Renault et al., 2005; Hazell et al., 2008; Hughes et al., 2010; Romero et al., 2010). In such systems it is vital that all temperature sensors (thermometers, thermocouples) are calibrated against a reference standard before use in experiments. This is important because the subsequent calibration of temperature within the exposure environment may require measurements from multiple sensors to assess the extent of heat transfer between the aluminium block and different locations within observed environment (Persoons et al., 2011).

This chapter describes the methods used to characterize the temperatures experienced by *N. lugens* during experiments designed to measure thermal activity thresholds.
2.3 Material and methods

The experimental system was based around the design described by Hazell et al. (2008), in which an ‘arena’ is milled out of an aluminium block with the temperature ‘controlled’ by fluid circulating through the block from a programmable alcohol bath (Haake Phoenix 11 P2; Thermo Electron Corp., Germany) as shown in Figure 2.1 (Hazell et al., 2008).

Figure 2.1 Aluminium block system for measuring insect activity thresholds; A, route for thermocouple to arena; B, observation arena; C, circulation channels for fluid from the alcohol bath (from Hazell et al., 2008).

Experiments were carried out in arenas of three sizes that differed in diameter, depth and surface area as follows: small arena (1.6 cm, 0.4 cm, 4.02 cm²), medium arena (2.5 cm, 0.8 cm, 11.19 cm²) and a large arena (4.0 cm, 0.8 cm, 22.62 cm²). In theory, when a cooled or heated fluid is pumped from an alcohol bath around the aluminium block, the temperature of the block should...
be equivalent to the temperature of the circulating fluid, although it may require a period of time for this equilibrium to be reached. Preliminary studies, however, indicated that whilst the temperature within the block was the same as cooled or heated fluid (and as set on the alcohol bath), the temperature measured in the middle of the arena was different to the block temperature. To investigate the pattern of temperature distribution across the experimental arenas, temperatures were recorded simultaneously at five locations as illustrated in Figure 2.2.

![Figure 2.2 Dimensions of arenas used to measure activity thresholds; 1-5 in Figure 2.2C indicate the positions of the five thermocouples in each arena.](image)

For all experiments, the temperatures within the aluminium block and in the arena were measured using type-K thermocouples (Tecpel CL – 326 DTM - 315) calibrated by the supplier against a digital thermometer with an accuracy of ±0.1°C (Heatmiser UK Ltd; Calibration report number: HM853-08/07/10DW). The calibration experiments were carried out in a controlled environment room at 23°C. To minimize the heat loss or gain to the room environment, the aluminium blocks were placed on a 25 mm deep polystyrene foam plate and enclosed within an insulating wrapping.
(Thermal Wrap, YBS Insulation). The top of the arena was covered by a thin sheet of Perspex within which five holes were drilled to allow access of the thermocouples into the arena (see Figure 2.2C).

In the first set of calibration experiments the five thermocouples were in contact with the base of the arena, referred to as the ‘surface temperature’; a second set of independent measurements were made in which the thermocouples were raised by 0.2 mm (for the small arena) and 0.4 mm (for the medium and large arena) off the base of the arena, and these values are described as the arena ‘ambient temperature’. The use of ‘fixed-point’ entry holes for the thermocouples through the Perspex arena cover ensured that the arena base and ambient temperatures were recorded at comparable positions both within and between experiments with different arenas. Both sets of arena temperatures were compared with the temperature measured within the body of the aluminium block, hereafter referred to as the reference temperature. Each experiment was initiated by either cooling or heating the aluminium blocks to one of six constant temperatures: 10, 15, 20, 30, 40, 50°C. The surface and ambient temperatures within the arena were measured after reaching a ‘steady state’ i.e. when there was no fluctuation in temperature over time, which was typically around 30 min after first reaching the set reference temperature. The experiments were repeated with three for each set temperature with each arena (n = 3).

The data were analysed by two-way ANOVA to determine differences between the arena surface and ambient temperatures and the reference temperature in arenas of different sizes. All data were also analysed by a simple linear regression to generate calibration equations for each arena size.
2.4 Results

In all arenas tested there was a significant difference between the surface temperatures and the reference temperature ($F_{10,72} = 1952.83$, $p < 0.001$, Figure 2.3), but this relationship was not affected by the size of the arena ($F_{10,72} = 2.94$, $p = 0.059$).

Figure 2.3 Relationship between arena surface temperatures in relation to the reference temperature in arenas of three sizes. A = small, B = medium and C= large. Measurement locations 1-5 in each arena are as coded in the figure.
There was also a significant difference between the ambient temperature in the arenas and the reference temperature ($F_{10,72} = 1128.17, p < 0.001$, Figure 2.4) and this relationship was significantly affected by arena size ($F_{10,72} = 3.28, p = 0.043$).

**Figure 2.4** Relationship between arena ambient temperatures in relation to the reference temperature in arenas of three sizes. A = small, B = medium and C = large. Measurement locations 1-5 in each arena are as coded in the figure.
The mean (± SE) of the arena surface and ambient temperatures in relation to the reference temperature in arenas of different sizes are shown in Table 2.1.

**Table 2.1** Mean (± SE) of arena surface and ambient temperatures in relation to the reference temperature at six constant temperatures in arena of different sizes.

<table>
<thead>
<tr>
<th>Reference temperature (°C)</th>
<th>Mean arena surface temperature (°C ± SE)</th>
<th>Mean arena ambient temperature (°C ± SE)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>small arena</td>
<td>medium arena</td>
</tr>
<tr>
<td>10</td>
<td>12.4±0.268</td>
<td>12.4±0.521</td>
</tr>
<tr>
<td>15</td>
<td>16.3±0.172</td>
<td>16.4±0.316</td>
</tr>
<tr>
<td>20</td>
<td>20.6±0.060</td>
<td>20.7±0.081</td>
</tr>
<tr>
<td>30</td>
<td>28.5±0.176</td>
<td>28.5±0.301</td>
</tr>
<tr>
<td>40</td>
<td>37.3±0.450</td>
<td>36.7±0.547</td>
</tr>
<tr>
<td>50</td>
<td>45.6±0.557</td>
<td>44.7±0.864</td>
</tr>
</tbody>
</table>

The data in Figures 2.3 and 2.4 and Table 2.1 were used to produce linear regression calibration graphs for arena surface (Figure 2.5) and ambient temperatures (Figure 2.6) in relation to the reference temperature for arenas of different sizes. The linear regression equations (all with very high R² values) can be used to predict the arena ambient and surface temperatures from the reference temperature that is displayed during the recording of insect movement at different temperatures (see Chapter 4 and 5).
Figure 2.5 Calibration graphs and equations for arena surface temperature and reference temperature in arenas of three sizes. For the small arena (A), $Y = 0.8322X + 3.8975$ ($R^2 = 0.997$), medium arena (B), $Y = 0.8072X + 4.3698$ ($R^2 = 0.992$) and large arena (C), $Y = 0.7672X + 5.0365$ ($R^2 = 0.978$).
Figure 2.6 Calibration graphs and equations for arena ambient temperature and reference temperature in arenas of three sizes. For the small arena (A), $Y = 0.7739X + 5.3846 (R^2 = 0.992)$, medium arena (B), $Y = 0.7482X + 5.9242 (R^2 = 0.978)$ and large arena (C), $Y = 0.7484X + 5.2351 (R^2 = 0.989)$. 
2.5 Discussion and conclusions

The system described in this chapter to assess critical thermal thresholds of insects is potentially subject to two main sources of error. Firstly, errors in temperature measurements due to the accuracy of the temperature sensors (Edney, 1951). This problem can be largely overcome by ensuring that all sensors, such as the thermocouples used in these experiments, are calibrated against a digital thermometer with a high level of accuracy (±0.1°C) (Heatmiser UK Ltd; Calibration report number: HM853-08/07/10DW).

The second source of error concerns the transfer of thermal energy between different substrates, in this case the aluminium block in which the temperature is determined by the temperature of the circulating fluid, and arena within the block which is subject to the influence of the wider environmental (room) temperature. In most thermal interactions between two substrates with different temperatures, the higher temperature of one decreases and the lower temperature of the other increases (McDermott et al., 1996; Marin, 2010), until the two interacting objects arrive at the same intermediate temperature, termed the thermal equilibrium (Bergethon and Simons, 1990). The aluminium block system, however, differs from this relationship because the arena temperatures are affected by an additional interaction, the air of the room within which the experiments are conducted (Keltner, 1998). This transfer of heat will continue as long as there is a difference in temperature between the arena and surrounding environment, resulting from differences in temperature between the arena and the fluid circulating around the aluminium block. Heat can move from one location to another (e.g. from the arena to wider controlled environment room by conduction, convection and radiation (Hilyard and Biggin, 1977; Recktenwald, 2006; Antar and Baig, 2009; Venkanna, 2010). In the aluminium block system heat
is transferred from the alcohol bath via the block to the arena and at thermodynamic equilibrium, heat transfer between these two substrates will be zero. However, the temperature differences between the arena and wider environment lead to the observed temperature differences between the arena and the aluminium block.

The data acquired from these experiments were used to calibrate the arena temperature in studies on chill and heat coma, as illustrated in Figure 2.7. For example, in the heat coma experiments, the aluminium block temperature was increased from 23°C to 50°C (Figure 2.7A). Insect movement was monitored throughout this increase in temperature using a digital video camera (Infinity 1-1; Lumenera Scientific, Canada) with a macro lens (Computar MLH-10X, CBC Corp., New York, NY) positioned over the arena and linked to a desktop computer. Data on insect movement and the temperature within the aluminium block (reference temperature) were recorded simultaneously by video recording software (Studio Capture DT; Studio 86 Designs, UK) as the temperature of the block was increased to 50°C. As the arena temperatures were however, progressively higher than room temperature as a result of the loss of heat energy to the surrounding environment, arena temperatures were lower than the ‘set’ reference temperature, and this difference increased as the temperature of the aluminium block increased above room temperature.

In chill coma experiments, the reverse situation occurs in which heat is gained from the wider environment such that the temperature in the arena will be higher than that of the aluminium block, and this difference will be greatest at the lowest set temperature (Figure 2.7B).
Figure 2.7 Illustration of heat transfer effects in experiments to assess high (A) and low (B) activity thresholds in insects. $\Delta T$ is temperature difference between the arena and surrounding environment.
The calibration data indicated that there was a difference between the set reference temperature and the surface and ambient temperatures within the observed arena at all temperatures from 10 to 50°C, and that this difference was greatest at the extremes of this range, reflecting the greater difference between the block and arena temperatures and the wider environmental temperature. There were also differences in these heat transfer relationships in arenas of different sizes, related to differences in surface areas (Muncaster, 1993). Thus, when the desired exposure temperature is higher (30 to 50°C) or lower (10 to 20°C) than the environmental (room) temperature, the larger arena will lose heat to the surrounding environment more rapidly than the medium and small arenas, and in general, will have a lower temperature that the other arenas, though this difference is relatively small (Table 2.1). These data provide a sound basis for selecting the most appropriate experimental set up for different species (Tong, 2001). For example, the small arena could be used for experiments with first instar nymphs of *N. lugens* and the medium or larger arena for adults, with each arena having its own calibration data.

In conclusion, these experiments have shown that measurement of temperature within the aluminium blocks used to investigate insect thermal thresholds are not representative of the surface or air temperatures experienced by organisms contained within the milled arenas of such systems; and the difference between the displayed and experienced temperatures is greatest at the extremes i.e. within the temperature ranges where the CT\textsubscript{min} and CT\textsubscript{max} are likely to fall. The experiments have shown that it is possible to determine the exposure temperature by calibration against a reference temperature, thus enabling accurate measurement of thermal thresholds.
CHAPTER 3

Relationship between Body Size and Dynamics of Thermal Equilibrium in Insects

3.1 Abstract

The ability of ectothermic organisms such as insects to maintain their body temperature ($T_b$) is an important contributory factor to their overall fitness. In experiments that measure critical thermal thresholds such as mobility and lethality it is vital that the exposure temperature and that of the sample or individual under observation are the same, so that the data acquired can be interpreted in a wider physiological and ecological context. The aim of this study was to examine the effect of insect body size on the time required to reach thermal equilibrium at different rates of temperature change and in different exposure environments. The species investigated (Liriomyza bryoniae, Nilaparvata lugens, and winged and wingless Calliphora vicina) had increasing body size. Individuals were warmed from 20 to 35°C in a programmable alcohol bath at three ramping rates (0.1, 0.5 and 1.0°C min$^{-1}$) in two types of container type commonly used in experiments on insect thermal biology. Mean lag time required to reach thermal equilibrium increased with body size and was also significantly affected by ramping rates and the containers used in the experiments (both $p < 0.001$). Mean lag time at 0.1°C min$^{-1}$ was significantly less than when the insects were warmed at 0.5 and 1.0°C min$^{-1}$, and when the experiments were carried out in glass tubes compared with plastic tubes within glass tubes. These results are discussed in relation to the methods used to measure insect thermal thresholds, and the use of such data in understanding the relationships between insects and temperature in natural environments.
3.2 Introduction

Living organisms are complex arrangements of different types of material (Patton, 1963; Hilyard and Biggin, 1977; Bergethon and Simon, 1990). The properties of these materials are specific to their function in the organism (Hilyard and Biggin, 1977; Campbell et al., 1999), but differences in these properties can lead to differences in key processes, such as the capacity to adjust to new environments, and this is particularly the case for ectothermic species that have limited ability to regulate their body temperature (Hilyard and Biggin, 1977). Insects are poikilotherms or cold-blooded animals in which the body temperature ($T_b$) is essentially the same as the environmental temperature ($T_e$). Many processes and behaviours in insects (development and reproduction activity) are strongly influenced by temperature (Heinrich, 1974; Knapp and Casey, 1986; Bakken, 1989; Heinrich, 1995; Speight et al., 1999; Chown et al., 2002; Angilletta Jr. et al., 2004), mainly because physiological functions are determined by the animal’s $T_b$ (Cokendolpher and Phillips, 1990; Nespolo et al., 2003; Chown and Terblanche, 2006; Bowler and Terblanche, 2008).

Thermoregulation is defined as the maintenance of the mean or variance of $T_b$ in relation to the mean or variance of the $T_e$ by behavioural, physiological or morphological strategies (Heinrich, 1974; May, 1977; May, 1979; Willot, 1997; Angilletta Jr. et al., 2006; Nice and Fordyce, 2006; Heinrich, 2007; Gullan and Cranston, 2010). Insects need these mechanisms to survive under conditions of thermal stress because of their limited ability to regulate $T_b$ (Bauwens et al., 1995; Bale and Hayward, 2010) and this is achieved by processes collectively known as capacity adaptation (Huey and Stevenson, 1979; Bennett and Huey, 1990; Huey and Berrigan, 2001; Kingsolver and Gomulkiewicz, 2003; Chown and Nicolson, 2004). Thermoregulation is one
aspect of homeostasis or a dynamic state of stability (May, 1977; May, 1979; Hertz et al., 1993; Maeda, 2005). Thermoregulatory mechanisms involve either change in metabolic heat production or in heat exchange with the environment (May, 1979; Casey, 1992; Walsberg, 1992; Purves et al., 2001; May, 2005). A number of studies have investigated heat exchange, the modes of heat transfer, and their application to animals (Clench, 1966; Thorkelson and Maxwell, 1974; Cena and Monteith, 1975; Bakken, 1976a Baken, 1976b; May, 1979; Dzialowski and O Conner, 2001; Voss and Reed, 2001). Heat exchange can be described by the laws of thermodynamics (May, 1979; Bergethon and Simons, 1990, Cummings et al., 2004; Angilletta Jr. et al., 2006) and involves conduction, convection, radiation and evaporation (May, 1979). According to the zeroth law of thermodynamics if there is no change in temperature when a sensor (e.g. thermometer) is placed in thermal contact with a ‘system’ (e.g. an organism), they are in a state of thermal equilibrium (Adkins, 1997). When an insect is placed in a new environment (e.g. controlled environment room or an alcohol bath) the $T_b$ is likely to be higher or lower than the new $T_e$ and will then decrease or increase over a period of time to be in thermal equilibrium with $T_e$ (Cummings et al., 2004).

This relationship has been observed in various experiments in which the cooling or heating source reaches the desired ‘set’ temperature before the insects within the exposure environment, although the cooling or heating source and the insect sample have the same initial temperature and are cooled or heated at the same rate to a new $T_e$. This difference in response time (‘lag time’) required to attain a new equilibrium state depends on the thermal properties of the materials (McNabb and Wake, 1991). A number of factors can influence an insect’s $T_b$ and its lag response time in changing thermal environments, including body size (Digby, 1955; Forsman,
2000; Davidowitz et al., 2003; Tanaka, 2005), the rate of temperature change, thermal properties of exposure containers, wing activity, and the ability of species to thermoregulate.

Knowledge of these interrelationships is important in the design of laboratory experiments (Chown and Sinclair, 2010). Measurements of insect $T_b$ under various environmental conditions have been made by several researchers (e.g. Edney, 1951; Waterhouse, 1951; Bakken, 1976b; Janusz, 1984; Turner and Lombard, 1990; Chown and Scholtz, 1993; Bauwens et al., 1995; May, 2005), including the effect of ramping rate on insect mortality (e.g. Evans, 1987; Neven, 1998; Ikediala et al., 2000; Dzialowski and O’Conner, 2001; Wang et al., 2002). In many studies, however, there is no reference to the time required for organisms to reach thermal equilibrium in different experimental systems and how this lag time is affected by the size of the specimen and the cooling or heating rates, which is an important consideration, as in most experiments of this type insects have to be held for a period of time before or after heating or cooling to allow equilibration of $T_b$ with $T_e$ (Terblanche et al., 2007). Whilst the main purpose of this investigation was to determine the lag time parameters for exposure of *Nilaparvata lugens*, the study included species that were smaller (*Liriomyza bryoniae*; Diptera: Agromyzidae) and larger (*Calliphora vicina*; Diptera: Calliphoridae) than the brown planthopper, and exposed samples in different containers and at different rates of warming.
3.3 Material and methods

3.3.1 Insect materials

The three species used in this study had been maintained in culture at the University for different periods of time prior to the experiments. *Liriomyza bryoniae* were reared on broad bean plants (*Vicia faba* var. Sutton Dwarf) in BugDorm-2120 ‘insect tents’ (Megaview Science Co. Ltd., Taiwan) and *N. lugens* on rice seedlings (*Oryza sativa* L. cv. TN 1) in clear plastic cages covered by a mesh lid (21 cm high and 6 cm diameter with 1.22 mm ventilation mesh). These two cultures were maintained in an insect quarantine room at 23±0.5°C, 16:8 L:D cycle. The blow fly, *C. vicina* was reared at 20°C, 18:6 L:D cycle in gauze-covered cages and received sugar and water *ad libitum*. All experiments were carried out on adult stages. To measure insect weights, 50 adults of each species were lightly anesthetized with carbon dioxide and weighed individually on a top loading electronic balance (Mettler AC 100, Mettler-Toledo Ltd., Beaumont Leys, Leicester, UK) to an accuracy of ± 0.0001 g. *Liriomyza bryoniae* were weighed in the same way in 10 replicates of 5 individuals to determine a mean individual weight.

**Table 3.1** Mean (± SE) weight and sources of insect materials.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mean body mass (g)</th>
<th>Source of culture</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. bryoniae</em></td>
<td>0.0005 ± 0.0001</td>
<td>Koppert Biological Systems B.V., The Netherlands</td>
</tr>
<tr>
<td><em>N. lugens</em></td>
<td>0.0013 ± 0.0001</td>
<td>Pulau Pinang, Malaysia (5° 23’N)</td>
</tr>
<tr>
<td><em>C. vicina</em></td>
<td>0.0272 ± 0.0014**</td>
<td>Birmingham, UK (52°N)</td>
</tr>
</tbody>
</table>
3.3.2 Measurement of surface-to-volume ratio

The accurate measurement of insect surface areas is difficult (Lockey, 1960) and usually cannot be achieved by direct observation of the living animal (Simanton, 1933). This problem is usually overcome by assuming that small organisms e.g. insects have a cube shape to make the calculation easier (Roberts and Ingram, 2001; Leggett, 2011). Using this approach, 50 adults of each species were lightly anesthetized with carbon dioxide and then photographed using a digital video camera (Infinity 1-1; Lumenera Scientific, Canada) with a macro lens (Computar MLH-10X, CBC Corp., New York, NY) positioned over the arena in the aluminium block system used to determine thermal thresholds (see Chapter 4 for details).

The digital images were processed using Image J (version 1.43u; http://rsb.info.nih.gov/ij) with measurements of length (L), width (W) and height (H) in millimetres. The equation for the surface area of a cube is \(2\times L \times W + (2\times L \times H) + (2\times W \times H)\) and for body volume is \(L \times W \times H\), where \(L, W\) and \(H\) are the length, width and height of the specimen respectively. The surface area is then divided by the volume to obtain the surface-to-volume ratio. Differences in surface-to-volume ratios between species were tested by an analysis of variance (ANOVA). Where significant differences were found, the data were further analysed by Tukey's honest significant difference post-hoc test.

3.3.3 Measurement of \(T_b\) and time required to reach thermal equilibrium

The times required for adults of each species to reach thermal equilibrium were assessed when individuals were warmed from 20 to 35°C at 0.1°C, 0.5°C, and 1.0°C min\(^{-1}\). Preliminary experiments indicated that \(C.\ vicina\) adjusted its \(T_b\) to reach thermal equilibrium at 35°C more rapidly than
other two species, and that the flies showed considerable ‘wing vibration’, raising the possibility that the insect body temperature was increased by a combination of the higher environmental temperature and thoracic muscle activity associated with flight. For this reason a comparison was made between the thermal equilibrium lag times of winged and wingless *C. vicina*. To obtain a sample of wingless flies, *C. vicina* were captured and lightly anesthetized with carbon dioxide and then the wings removed with a pair of scissors, close to the thorax.

The $T_b$ of individual insects was measured by attaching a 0.1 mm diameter copper-constant K type thermocouple (Tecpel CL–326 DTM-315) on to the ventral surface of the thorax with petroleum gel. Because of their larger body size, *C. vicina* were captured and lightly anesthetized with carbon dioxide to facilitate handling before attaching the thermocouple. The insects attached to the thermocouples were placed in either an Eppendorf tube (1.5 ml, SARSTEDT, Aktiengeselischaft & Co. for *L. bryoniae* and *N. lugens*) or a polystyrene tube (30 ml for winged and wingless *C. vicina*), both within a glass tube, or directly into a glass tube (100 ml, Pyrex), such that the specimens were individually ‘suspended’ within the ‘air environment’ of the tubes but without contact with any surface (Figure 3.1). In the Results section, the Eppendorf and polystyrene tubes are described collectively as ‘plastic tubes’. The tubes containing the insects were placed in a programmable alcohol bath (Haake Phoenix 11 P2; Thermo Electron Corp., Karlsruhe, Germany) and held at 20°C for 30 min. The samples were heated to 35°C at 0.1°, 0.5°, and 1.0°C min$^{-1}$ with the insect $T_b$ measured at one minute intervals; 35°C was selected as the new $T_e$ as it was known that all species could survive at this temperature, hence the $T_b$ and thermal lag times obtained would be for live insects. Simultaneous temperature measurements were made at one minute intervals in blank plastic tubes within glass tubes (Figure 3.1A) and in
blank glass tubes (Figure 3.1B) and of the fluid in the alcohol bath. Insect $T_b$ was recorded until it reached the $T_e$ (35°C). Lag times were calculated as the time required for the insect $T_b$ to reach thermal equilibrium with the temperature of the fluid in the alcohol bath for a sample of 30 individuals of $L. bryoniae$, $N. lugens$ and winged and wingless $C. vicina$ in plastic tubes within glass tubes and directly within glass tubes.

Mean lag times of the different species within each heating rate were analysed by one-way ANOVA and significant differences identified by Tukey's honest significance difference post-hoc test. The relationships between lag times, ramping rates and exposure containers were analysed by a two-way ANOVA.

**Figure 3.1** Experimental design for measuring body temperatures of insects when exposed in A, plastic tubes within glass tubes, and B, direct exposure within glass tubes.
3.4 Results

3.4.1 Surface-to-volume ratios

The mean surface-to-volume ratios (± SE) for *L. bryoniae*, *N. lugens* and *C. vicina* were 8.31±0.16, 4.31±0.11 and 1.59±0.05 mm⁻¹ respectively (Figure 3.2) and all differences between species were significant (p < 0.001, $F_{2,147} = 898.47$).

![Mean surface-to-volume ratio](image)

**Figure 3.2** Mean surface-to-volume ratio (± SE) in *L. bryoniae*, *N. lugens* and *C. vicina*. Mean values with the same letter are not significantly different (p ≤ 0.05); n = 50 for each species.
3.4.2 Effect of body size on lag times

Mean (± SE) lag times varied among species and between winged and wingless *C. vicina* (Figure 3.3). When exposed as individuals in a plastic tube within a glass tube (Figure 3.3A) or in glass tube (Figure 3.3B) a similar pattern was observed at all ramping rates in which the smallest species heated up faster than the larger insects with mean lag times in the order of *L. bryoniae* < *N. lugens* < winged *C. vicina* < wingless *C. vicina* respectively. The mean lag times required for *L. bryoniae*, *N. lugens*, winged *C. vicina* and wingless *C. vicina* to reach the target temperature (35°C) when exposed in plastic tubes within glass tubes were 4.41±0.47, 5.28±0.41, 10.80±1.11 and 24.45±1.09 min at 0.1°C min⁻¹, 8.19±0.49, 8.96±0.96, 9.71±1.28 and 30.55±1.75 min at 0.5°C min⁻¹, and 14.55±1.11, 21.79±1.01, 16.23±1.36 and 53.63±4.40 min for testing at 1.0°C min⁻¹ (Figure 3.3A). The equivalent mean lag times for *L. bryoniae*, *N. lugens*, winged *C. vicina* and wingless *C. vicina* exposed in glass tubes were 2.97±0.30, 4.53±0.83, 5.81±1.09 and 16.63±1.33 min at 0.1°C min⁻¹, 6.96±0.48, 8.53±0.54, 8.61±1.05 and 21.84±1.01 min at 0.5°C min⁻¹, and 8.24±0.35, 15.31±1.05, 11.26±1.50 and 37.85±2.34 min at 1.0°C min⁻¹ (Figure 3.3B).

When warmed at 0.1°C min⁻¹ the mean lag times to reach thermal equilibrium at 35°C were significantly longer in wingless *C. vicina* than *L. bryoniae*, *N. lugens*, and winged *C. vicina*, both in plastic tubes within glass tubes (F₃,₃₆ = 121.47, p < 0.001) and in glass tubes (F₃,₃₆ = 42.87, p < 0.001). Also, wingless *C. vicina* took significantly longer to adjust its T_b than *L. bryoniae*, *N. lugens*, and winged *C. vicina* in plastic tubes within glass tubes (F₃,₃₆ = 80.11, p < 0.001) and in glass tubes (F₃,₃₆ = 73.25, p < 0.001) at a ramping rate of 0.5°C min⁻¹. However, there were no differences among *L. bryoniae*, *N. lugens*, and winged *C. vicina* at 0.1 and 0.5°C min⁻¹ in either type of exposure container.
At the fastest rate of warming (1.0°C min⁻¹) the lag times of wingless *C. vicina* and *N. lugens* were significantly longer than *L. bryoniae* and winged *C. vicina* in the plastic tubes within glass tubes (F₃,₃₆ = 57.26, p < 0.001) and in glass tubes (F₃,₃₆ = 80.56, p < 0.001).

**Figure 3.3** Effect of insect body size on mean lag times (± SE) when exposed in plastic tubes within glass tubes (A) and in glass tubes (B). White bars represent *L. bryoniae*, black bars *N. lugens*, cross-hatch bars winged *C. vicina* and dotted bars wingless *C. vicina*. At 0.1°C min⁻¹ mean values with the same lower-case letter are not significantly different (p ≤ 0.05); at 0.5°C min⁻¹ mean values with the same lower-case letter followed by the same number are not significantly different (p ≤ 0.05) and at 1.0°C min⁻¹ mean values with the same capital letter are not significantly different (p ≤ 0.05). N = 30 at each ramping rate.
3.4.3 Effect of ramping rates on lag time

The mean lag times to reach thermal equilibrium were significantly longer when the temperature of the alcohol bath was increased at 1.0°C min⁻¹ compared with 0.5 and 0.1°C min⁻¹ ($F_{2, 117} = 16.109$, $p < 0.001$) with a similar result with plastic tubes within glass tubes (Figure 3.4A) and in glass tubes (Figure 3.4B). At heating rates of 0.1 and 0.5°C min⁻¹ lag times were similar between *N. lugens* and winged wingless *C. vicina* (experiments carried out in plastic tubes within glass tubes, Figure 3.4A), and winged and wingless *C. vicina* when exposed in glass tubes (Figure 3.4B). Likewise, there was no difference in lag time between *L. bryoniae* and wingless *C. vicina* when heated at 0.5 and 1.0°C min⁻¹ (see Figure 3.4B).
Figure 3.4 Effect of ramping rates on the mean lag time (± SE) when exposed in plastic tubes within glass tubes tubes (A) and in glass tubes (B). White bars represent a ramping rate of 0.1°C min⁻¹, black bars 0.5°C min⁻¹ and cross-hatch bars 1.0°C min⁻¹. At 0.1°C min⁻¹ mean values with the same lower-case letter are not significantly different (p ≤ 0.05); at 0.5°C min⁻¹ mean values with the same lower-case letter followed by the same number are not significantly different (p ≤ 0.05) and at 1.0°C min⁻¹ mean values with the same capital letter are not significantly different (p ≤ 0.05). N = 30 at each ramping rate.
3.4.4 Effect of exposure containers on lag time

All species took longer to reach thermal equilibrium when exposed in plastic tubes within glass tubes than directly in glass tubes (Figure 3.5). For *L. bryoniae* mean lag times differed significantly at 0.1 (F_{1, 18} = 6.68, p = 0.019) and 1.0°C min^{-1} (F_{1, 18} = 29.53, p < 0.001) respectively, but not at 0.5°C min^{-1}.

Mean lag times of *N. lugens* in plastic tubes within glass tubes and in glass tubes were only significantly different at 1.0°C min^{-1} (F_{1,18} = 19.86, p < 0.001).

The results for winged *C. vicina* followed a similar pattern to that of *L. bryoniae* with significantly different lag times between plastic tubes within glass tubes compared with glass tubes at 0.1 (F_{1, 18} = 10.84, p = 0.004) and 1.0°C min^{-1} (F_{1, 18} = 6.01, p = 0.025) respectively, but not at 0.5°C min^{-1}.

The mean lag times of wingless *C. vicina* exposed in plastic tubes within glass tubes and in glass tubes were significantly different at all the ramping rates: 0.1 (F_{1, 18} = 20.65, p < 0.001), 0.5 (F_{1, 18} = 18.69, p < 0.001) and 1.0°C min^{-1} (F_{1, 18} = 10.02, p = 0.005) respectively.

From the two way ANOVA analysis, mean lag times were significantly affected by ramping rates (F_{2, 212} = 22.77, p < 0.001) and exposure containers (F_{2, 212} = 11.42, p < 0.001). The interaction between ramping rate and container type also significantly affected mean lag times (F_{2, 212} = 8.91, p < 0.001).
Figure 3.5 Effect of type of exposure container on mean lag times (± SE) at ramping rates of 0.1 (A), 0.5 (B) and 1.0°C min⁻¹ (C). White bars represent experiments carried out in plastic tubes within glass tubes and grey bars in glass tubes. Mean values with the same lower case letter (L. bryoniae), lowercase letter followed by the same number (N. lugens), capital letter (winged C. vicina) and capital letter followed by the same number (wingless C. vicina) are not significantly different (p ≤ 0.05). N = 30 for each species in each type of container.
3.5 Discussion and conclusions

Biological systems are complex because of the interdependency and interaction between chemical and physical forces (Bergethon and Simons, 1990). The properties of animals that influence the exchange of energy and heat flux include the rates of metabolism and evaporative loss (Casey, 1992; Voss and Reed, 2001) and these in turn affect animal thermoregulation capacity (Porter and Gates, 1969; Walters and Hassall, 2006). These interrelationships are important in studies of the thermoregulatory behaviour and responses of ectothermic organisms such as insects; and without knowledge of the interactions between factors such as surface-to-volume ratios, rates of temperature change and properties of the exposure environment, the design of experiments may be flawed and the values obtained inaccurate, and in both cases, the data will be of limited value in understanding the ecophysiology of species in natural environments. The studies reported in this chapter investigated the effect of differences in insect body size, warming rate and exposure container on the time taken for organisms to reach thermal equilibrium with a temperature-controlled and confined experimental situation (alcohol bath) commonly used in the study of insect thermal biology.

The first main conclusion to draw from these studies is that body size has a significant influence on the time required to achieve thermal equilibrium and that smaller species reach this state most quickly (Figure 3.2). These differences in lag times are attributable to differences in the surface-to-volume ratios (Planinšič and Vollmer, 2008). Thus insect $T_b$ is determined not only by $T_e$, but also by factors such as metabolic heat production, insolation and heat loss by means of convection, conduction, radiation and evaporation (Waterhouse, 1951; Digby, 1955; Porter and Gates, 1969; May, 1979; Casey, 1992; Pereboom and Biesmeijer, 2003). All of these factors are
influenced by body size because thermal energy in living organisms is proportional to body volume since the heat is generated internally and heat loss is proportional to the surface area (Porter and Gates, 1969; Stevenson, 1985; Casey, 1992).

The surface-to-volume ratio is an important concept in biology (Simanton, 1933; Davidowitz et al., 2003; Radtke and Williamson, 2005). Although *L. bryoniae*, *N. lugens* and *C. vicina* have different sizes, they are all composed of cells of approximately the same size, 10-30 µm in diameter (Machalek, 2005), but larger organisms will contain more cells (Silverstein et al., 2002). The simplest conceptual model of the cell is of a physio-chemical system that interacts with its environment to exchange mass and energy at a rate related to its metabolism (Barford, 1995), and the rate of metabolism is related directly to the volume of the cell (Robertson, 2009; Starr et al., 2010). The relationship between metabolism and body weight is however, not linear because body heat can be either lost or gained from the external environment (Robertson, 2009; Mills, 2010). As the size of an organism increases, its volume increases more than its surface area. Thus, the surface-to-volume ratio decreases as organisms increase in size (Vaughan et al., 2011). The greater surface-to-volume ratio of *L. bryoniae* compared with *N. lugens* and *C. vicina* (Figure 3.2) thus explains why *L. bryoniae* has the shortest lag time as their proportionally higher surface area enables them to gain heat more quickly (Robertson, 2009; Starr et al., 2010).

Mean lag time also differed significantly between winged and wingless *C. vicina* and was shorter when the wings were in place and functional. The more rapid acquisition of thermal equilibrium is therefore most likely a combination of the higher external temperature and body heat produced by wing beating. In insects with strong flight capacity such as butterflies, bumblebees, beetles, flies, dragonflies, the thoracic flight muscles are relatively large and oscillate at high frequencies.
in preparation for flight (Heinrich, 2007, Matthews and Matthews, 2010), thus increasing the body temperature (Newport, 1837; Heinrich, 1974; Chapman, 1998; Heinrich, 2007). Janusz (1984) reported that at least 80% of the energy produced by contraction of the flight muscles appears as heat and this leads to endogenous heat generation. However, the effect of flight muscle activity on the T\textsubscript{b} of small insects, such as \textit{L. bryoniae} and \textit{N. lugens}, is usually insignificant because of the small size of the muscles and the high rate of the heat loss from the organisms (Chapman, 1998). A second contributory factor to this internal heat generation may be related to blood circulation around the wings of \textit{C. vicina}. Clench (1966) reported that wings are the principal structures used in insect thermoregulation. Thus when the wings are exposed to the sun or a source of higher temperature, the blood gains heat from solar radiation or external environment which is then carried into the body i.e. the wings function as ‘heat exchangers’.

A number of previous studies have highlighted the effect of body size on thermoregulation. For example, in stingless bees, smaller bees reach lower temperatures and warm up and lose heat more rapidly than larger bees (Pereboom and Biesmeijer, 2003). Other studies have suggested that differences in size may affect the ability to thermoregulate and play a role in thermal niche partitioning and geographical distribution patterns (Verdú et al., 2006). Verdú et al. (2006) reported that prior to flight, larger dung beetles (>1.9 g) elevate and maintain their T\textsubscript{b} at levels well above T\textsubscript{e} whereas in smaller beetles (<1.9 g), the T\textsubscript{b} tends to conform with T\textsubscript{e}. With the knowledge that body size can affect rates of heat exchange, this may be a consideration when there is difference in size between the sexes. For example, Forsman (2000) found that the preferred T\textsubscript{b} in males and females of pygmy grasshoppers (\textit{Tetrix subulada}) were significantly different and this may be related to the thermal properties of different body sizes.
In this study the times required to reach thermal equilibrium were also significantly affected by the rates of temperature change between 20 and 35°C ($F_{2, 212} = 22.779, p < 0.001$); slower ramping rates reduced the lag time before the $T_b$ was the same as the $T_e$. In winged *C. vicina*, lag times are however, not so clearly related to ramping rates, which may reflect the contribution of wing vibration to $T_b$ in this species. The fact that thermal equilibrium is reached more quickly at slower ramping rates is probably a consequence of effects on metabolism (Evans, 1987; Stephens et al., 1994; Neven, 1998; Wang et al., 2002). Whilst there have been no previous studies on the thermal lag times of these three species, the results obtained can be compared with similar data for other species. For example, several authors have measured the effect of temperature ramping rates on insect mortality (Alderson et al., 1998; Neven, 1998; Ikediala et al., 2000) and found that at slower heating rates, longer exposure times were required to achieve the same level of mortality (Feder et al., 1997; Chown and Nicolson, 2004). Terblanche et al. (2007) also reported that rates of temperature change affect the measurement of insect critical thermal maximum and minimum temperatures. The feature that is different about the current study is the focus on the effect of ramping rates on the lag time of different species to achieve equilibrium at a new $T_e$. Whilst most studies expose insects directly to the assay temperature with equilibration assumed to occur in less than a minute (Hoffmann et al., 1997; Chown and Nicolson, 2004), the data reported here indicate that lag times are often longer and are affected by both the size of the species and the rate at which the organisms are heated or cooled.

In addition, the type and number of containers within which insects are confined is apparently rarely considered in thermal biology experiments. When a sample of insects is exposed in plastic tubes within a glass tube and directly within a glass tube and then immersed into fluid with a
higher temperature, there will be a transfer of thermal energy (Mattos and Gasper, 2002). In this study, insects in which the $T_b$ was measured in a plastic tube within a glass tube took longer to reach thermal equilibrium than individuals in glass tubes alone. This can be explained by the fact that heat energy would be transferred from the alcohol through the wall of either one or two substrates before reaching the insect. Thus the number of containers and their thermal properties affect the time taken before the $T_b$ equates to the $T_e$.

In summary, the experiments described in this chapter have highlighted a number of factors that can influence the speed with which insects achieve thermal equilibrium when experiencing a change in temperature, whether gradual (and at different rates) or more abruptly. The key message to emerge is that unless these factors are considered in the design of experiments, the data obtained may be inaccurate and unreliable.
CHAPTER 4

Upper Thermal Thresholds of the Brown Planthopper *Nilaparvata lugens*

4.1 Abstract

The brown planthopper *Nilaparvata lugens* is the most serious pest of rice across the world, especially in tropical climates. *Nilaparvata lugens* nymphs and adults were exposed to high temperatures to determine their critical thermal maximum (CT\textsubscript{max}), heat coma temperature (HCT) and upper lethal temperature (ULT). Thermal tolerance values differed between developmental stages: nymphs were consistently less heat tolerant than adults. The mean (± SE) CT\textsubscript{max} of nymphs and adult females and males were 34.9 ± 0.3, 37.0 ± 0.2 and 37.4 ± 0.2°C respectively, and for the HCT were 37.7 ± 0.3, 43.5 ± 0.4 and 42.0 ± 0.4°C. The ULT\textsubscript{50} values (± SE) for nymphs and adults were 41.8 ± 0.1 and 42.5 ± 0.1°C respectively. The results indicate that nymphs of *N. lugens* are currently living at temperatures close to their upper thermal limits. Climate warming in tropical regions and occasional extreme high temperature events are likely to become important limiting factors affecting the survival and distribution of *N. lugens*. 
4.2 Introduction

Temperature has a direct influence on many life history parameters of insects (Angilletta Jr. et al., 2002; Walther et al., 2002; Root, 2003; Hanna and Cobb, 2007; Tewksbury et al., 2008). A large number of studies have been conducted over the past 20-30 years to investigate the effects of predicted scenarios of climate warming on insects (Hill et al., 2002; Wilson et al., 2005; Deutsch et al., 2008). Much of this research has focused on the effects of increases in summer temperatures of 1-2°C on rate-based processes of experimental populations, and mainly in polar and temperate climates (Tewksbury et al., 2008; Parmesan et al., 1999; Bale et al., 2000; 2001; Karban and Strauss, 2004; Musolin, 2007), or by the monitoring of shifts in distributions that have been correlated with natural climate warming (Gutierrez et al., 2008). Also, whilst cold tolerance has been an area of research interest since the pioneering studies of Salt (Block et al., 1990; Chown and Nicolson, 2004), there has been less focus on the high temperature tolerance of insects, especially those living in tropical areas, or on the proximity of their upper thermal limits to current and future temperature regimes. This may be explained by the assumption that insects already living in high temperature environments may be less affected by increases in temperature than species inhabiting cooler climates, or that they have the ability to cope with such changes (Bale and Hayward, 2010). This assumption cannot however, be tested without accurate information on the thermal limits of tropical insects which can then be compared with data on current and predicted maximum temperatures. It is known that relatively small increases in temperature may become lethal or sub-lethal for such species (Talekar and Shelton, 1993; Klok et al., 2004; Lapointe et al., 2007).
When an insect is progressively warmed to high temperature, a sequence of distinct observable or measureable events occurs (Bowler, 2005; Folk et al., 2007; Hazell et al., 2010a). Firstly, the specimen moves in an increasingly uncoordinated way and becomes immobile; this is the critical thermal temperature ($CT_{\text{max}}$). As the temperature is further increased, all small-scale movement of appendages (legs, antennae) ceases as the organism enters a state of ‘heat coma’ (HCT), after which, at a higher temperature, the insect dies at its upper lethal temperature (ULT) (see Hazell et al., 2010a for a description of these physiological states).

The interrelationships between these three indices are of interest because they provide a physiological insight to events of ecological importance. For example, on a local scale, at the $CT_{\text{max}}$ insects are unable to move and hence to locate new food resources or escape from predators (Hanna and Cobb, 2007), and on a wider scale, such responses will affect distributions and potential range expansion (Bale et al., 2002; Gullan and Cranston, 2010; Romero et al., 2010); and these indices vary between different life cycle stages within a species (Krebs and Loeschcke, 1996). Also, although the $CT_{\text{max}}$ and heat coma occur at lower temperatures than the ULT, it is known that for some species heat coma is irreversible and therefore the insect is effectively dead at this temperature (Huey and Stevenson, 1979; Fischer et al., 2010).

Previous studies on the high temperature tolerance of tropical insects have investigated $CT_{\text{max}}$ and heat coma temperature (Heath et al., 1971; Gaston and Chown, 1999; Renault et al., 2005; Terblanche et al., 2008), ULT (Addo-Bediako et al., 2000; Chown, 2001; Chidawanyika and Terblanche, 2011) and heat shock proteins (Feder and Krebs, 1998; Krebs and Feder, 1998; Robertson, 2004; Klose et al., 2008). These studies have investigated species of African, South American or European origin with less known about species from Asia.
In this study, the focus is on the brown planthopper *Nilaparvata lugens* (Stål). *Nilaparvata lugens* is a major pest of rice throughout Asia causing serious yield losses in many countries (Cuong et al., 1997). *Nilaparvata lugens* has a high migratory ability by wind-assisted flight and high reproductive capacity (Kisimoto, 1979). Seo et al. (2009b) report that during the rice growing season *N. lugens* migrates every year on south-westerly airflows from the south-east of China to South Korea. Fluctuation of *N. lugens* population abundance in rice fields is highly correlated with temperature (Win et al., 2011). However, as with many tropical species, there is a lack of information about the high temperature tolerance of *N. lugens* and therefore the likely effects of climate warming on this important species. Thus, the aim of this study was to characterize the high temperature tolerance of nymphs and adults of *N. lugens* via CT\(_{\text{max}}\), HCT and ULT, and then compare these data with information on maximum environmental temperatures across the distribution of *N. lugens* in current and future predicted climates.

### 4.3 Material and methods

#### 4.3.1 Insect cultures

Adults of *N. lugens* were provided by MARDI (Malaysian Agricultural Research and Development Institute) Station at Pulau Pinang, Malaysia and maintained in a quarantine room at 23 \(\pm\) 0.5\(^\circ\)C, 16:8 L:D cycle on rice seedlings (*Oryza sativa* L. cv. TN 1) within individually sealed containers (transparent plastic cylinder, 21 cm high and 6 cm diameter with 1.22 mm ventilation mesh). This rice cultivar does not contain any major resistance genes to brown planthopper and is often used as a susceptible control in studies on plant resistance (Cuong et al.,...
1997). The seedlings were used at the maximum tillering stage and replaced every 4-5 days or when there were any signs of deterioration. All experiments were carried out with first instar nymphs (24-48 h old) and unmated adults (30–35 days old). In experiments carried out on adults, newly hatched first-instar nymphs were reared together until the late fifth instar after which males and females were selected and reared separately to obtain unmated adults.

4.3.2 Determination of $CT_{\text{max}}$ and HCT

The $CT_{\text{max}}$ and HTC were determined using a method modified from Hazell et al. (2008). Insects were monitored within an arena in an aluminium block attached to an alcohol bath. The initial temperature within the arena was set at 20°C. A sample of 10 first instar nymphs, adult females or males was allowed to settle for 15 min after which the temperature was increased at 0.5°C min$^{-1}$ up to 35°C. Thereafter, the temperature within the arena was increased from 35 to 55°C at 0.1°C min$^{-1}$ so as to minimise the chance of any ‘heat hardening’ response during warming (Hazell et al., 2010a).

Movement behaviour of *N. lugens* was viewed using a digital video camera (Infinity 1-1; Lumenera Scientific, Canada) with a macro lens (Comuptar MLH-10X, CBC Corp., New York, NY) positioned over the arena and linked to a desktop computer. Data on insect movement and temperature within the arena were recorded simultaneously by video recording software (Studio Capture DT; Studio 86 Designs, UK). The $CT_{\text{max}}$ was defined as the temperature at which the insect ceased coordinated movement and became immobile; the HTC was the temperature at which the last movement of an appendage (antenna, leg) occurred. Each experiment was repeated with a further sample of 10 individuals of each life cycle stage ($n = 20$).
4.3.3 Determination of ULT

The upper lethal temperature is usually determined by exposing insects to increasingly higher temperatures and recording the mortality at each temperature. The crucial factor is that the ULT is expressed as the temperature at which mortality occurs after a brief exposure (seconds or a few minutes), though death may occur post-exposure, hence estimates of mortality are usually made some days later (Hazell et al., 2010a). Other experimental formats examine the effect of the duration of exposure on the ULT or the ability to rapidly heat harden (Chown and Nicolson, 2004; Terblanche et al., 2007).

A key requirement in ULT experiments is that the insects should actually experience the desired exposure temperatures allowing for the time lag in heat transfer from the exposure environment to the sample, which will be longer in larger species (see chapter 3). A failure to take into account the time required for insects to reach thermal equilibrium with their exposure environment can lead to errors in the assessment of the ULT (Walsberg and Wolf, 1996).

For all ULT experiments, 10 first instar nymphs, adult males or females were placed in a 0.9 ml Eppendorf tube (with five replicates at each exposure temperature), and then placed at the bottom of a glass test tube suspended in a programmable alcohol bath (Haake Phoenix 11 P2; Thermo Electron Corp., Germany with temperature accuracy of ± 0.5°C). The samples were held at 20°C for 30 min to reduce stress associated with handling and then heated to a range of temperatures at 0.5°C min⁻¹. When the temperature in the alcohol bath reached the target temperature, the insects were held at this temperature for a period of time to ensure that all of the sample experienced the required temperature; preliminary experiments indicated this was 2 and 6 min for nymphs and
adults respectively. Thereafter, all samples were ‘cooled’ to the rearing temperature at 0.5°C min⁻¹ and then transferred to recovery trays (transparent plastic boxes, 16 x 8.5 x 28 cm³ with 1.22 mm ventilation mesh) containing rice plants and kept at 23°C, 16:8 L:D. Mortality was assessed 72 h after exposure. The data were analyzed by Probit in Minitab 15 (Minitab Inc., 2007) to estimate the temperature at which 50% of the sample was killed, the ULT₅₀. The controls revealed 99% survival.

An analysis of variance (ANOVA) was used to compare mean data between life cycle stages with 95% confidence limits. Where significant differences occurred, the data were further analysed by Tukey's honest significance difference post-hoc test to separate statistically heterogenous groups.

4.4 Results

4.4.1 CT_max and HCT

The mean CT_max (± SE) were 34.9 ± 0.3, 37.0 ± 0.2 and 37.4 ± 0.2°C for nymphs and adult females and males respectively (Figure 4.1A) with temperature ranges of 30-36°C, 34-38°C, and 35-38°C for the three life cycle stages (Figure 4.2A). The CT_max was significantly lower in first instar nymphs than adults (F2,27 = 33.55, p < 0.001), but not between the sexes.
Figure 4.1 Thermal activity thresholds of different life cycle stages and sexes of *N. lugens*. Mean (± SE) $\text{CT}_{\text{max}}$ (A) and HCT (B). Mean values with the same letter are not significantly different ($p \leq 0.05$); $n = 20$ for first instar nymphs, adult females and males.

The mean HCT (± SE) of nymphs, females and males were 37.7 ± 0.3, 43.5 ± 0.4 and 42.0 ± 0.4°C respectively (Figure 4.1B), with temperature ranges of 35-39°C, 39-46°C, and 39-44°C (Figure 4.2B). The HCT of nymphs was significantly lower than the adult morphs ($F_{2, 27} = 68.21$, $p < 0.001$), and also between the sexes ($p = 0.013$), with females having the higher HCT. Insects that entered heat coma were unresponsive to stimuli and found to be dead when cooled to a lower temperature.
Figure 4.2 Temperature range of thermal activity thresholds of different life cycle stages and sexes of *N. lugens*. Changes in the CT$_{\text{max}}$ (A) and HCT (B) for first instar nymphs (white bars), adult females (cross-hatch bars), and adult males (black bars); n = 20 for each life cycle stage.

4.4.2 ULT

The mean ($\pm$ SE) ULT$_{50}$ of the first instar nymphs (41.8 ± 0.1°C) was significantly lower than for adults (42.5 ± 0.1°C), ($F_{1,8} = 17.52$, $p = 0.003$, Figure 4.3). The ULT was higher than the HCT of nymphs (37.7°C) but similar for adults (HCT of 43.5° and 42°C for females and males respectively).
**Figure 4.3** Mean (± SE) ULT$_{50}$ of first instar nymphs and adults of *N. lugens*. Mean values with the same letter are not significantly different (p ≤ 0.05); n = 50 at each exposure temperature.

**4.5 Discussion and conclusions**

Climate, particularly temperature, is known to exert a strong influence on the distribution and abundance of species, often through effects on mortality (Parmesan, 1996; Davis et al., 1998; Hodkinson, 1999; Walther, 2002; Thomas et al., 2004; Wilson et al., 2005; Kerr et al., 2007; Terblanche et al., 2008). It is also known that the sequence of thermal events from immobility to death occurs over a narrower range at high than at low temperatures (Hazell et al., 2008; Hazell et al., 2010a). Whilst some studies have shown that insects can recover from exposure at their heat
coma temperature, for other species the heat coma state is irreversible and usually leads to death (Hazell et al., 2010a). This was the case with *N. lugens* as in this study where there was no recovery from heat coma after transfer to a lower temperature. Furthermore, heat tolerance is usually increased by much less than cold tolerance when insects are reared in an acclimation regime (Hazell et al., 2010b). Measurements of the CT$_{\text{max}}$, heat coma and ULT of tropical insects therefore provide a basis for assessing the likelihood of thermal stress under current climate conditions and the risk posed by higher temperatures under different scenarios of climate warming.

The results from this study suggest that differences in body size and volume affect heat tolerance; thus the CT$_{\text{max}}$, heat coma temperature and ULT$_{50}$ of nymphs was consistently and significantly lower than that of adults, and for one of these indices (heat coma), adult males were less heat tolerant than females. Such differences between juvenile and adult insects has been previously reported (Chapman, 1998). The ratio of surface area-to-volume is greater for nymphs than adults (Casey, 1992) and as the gain and loss of heat from and to the external environment by processes including mixed convection and radiation (Hilyard and Biggin, 1977; Casey, 1992; Recktenwald, 2006) are proportional to surface area (Stevenson, 1985), heat transfer occurs more rapidly in nymphs with resultant lower thermal indices. Whilst these data indicate that adults are generally more heat tolerant than nymphs, in terms of population viability over successive generations, success will be largely dependent on the limits imposed by the least thermally tolerant life cycle stage i.e. the higher heat tolerance of adults is ecologically irrelevant if the nymphal stages are dead or destined to die.
The critical information derived from this study indicates that some first instar nymphs become immobilized by heat stress at around 30°C and among the more heat tolerant adult stage, no insects were capable of coordinated movement at 38°C. There was no recovery after entry into heat coma, at temperatures around 38°C for nymphs and 42-43°C for adults. In similar studies the cicada *Magicicada cassini* was unable to maintain coordinated movement above 43°C but could recover from exposure at this temperature (Heath et al., 1971). This recovery ability contrasts with *N. lugens* and other species (Hazell et al., 2010a), but may be related to the inability in earlier studies to distinguish accurately between the CT$_{\text{max}}$ and heat coma temperatures. Renault et al. (2005) reported differences in the CT$_{\text{max}}$ of first instar larvae of three species of Coleoptera ranging from 45.6°C in *Osmoderma eremite* to 48.5°C in *Gnorimus nobilis* and 51.4°C in *Cetonischema aeruginosa*, all of which are higher than that of *N. lugens*. CT$_{\text{max}}$ values are ecologically important because they represent the effective limit to coordinated movement behaviour within the thermal tolerance range of a species and life cycle stage (Bursell, 1964).

Within this range, an insect’s physiological responses increase with temperature to an optimum and then rapidly decrease through the effects of heat stress (Huey and Bennett, 1990; Terblanche et al., 2007).

Insects use various behavioural mechanisms to avoid the extremes of heat stress (Purves et al., 2001; Wharton, 2002) including movement to more shaded locations such as the underside of leaves (Turnock, 1999), burrowing into the soil, which is common in desert species (Gullan and Cranston, 2010), or restricting activity to cooler periods within the diurnal cycle (Yang et al., 2008). All of these responses, however, need to be anticipatory, because progression past the optimum temperature to the CT$_{\text{max}}$ and HCT will limit the ability of insects to move to more
favourable thermal sites, and as a result, to locate resources such as food, mates and oviposition sites, and escape from natural enemies (Hanna and Cobb, 2007; Romero et al., 2010).

At 41.8°C and 42.5°C respectively, approximately 50% of nymphs and adults of *N. lugens* are killed in exposures of only 2 and 6 min. The ULT$_{50}$ of the tsetse fly, *Glossina pallidipes* was 37.9°C, 36.2°C and 35.6°C respectively in exposures of 1, 2 and 3 h (Terblanche et al., 2008) and Chidawanyika and Terblanche (2011) found that ULT$_{50}$ of adult codling moth *Cydia pomonella* was 44°C in a 2 h exposure. These data indicate a broad similarity in ULT$_{50}$ values between species (more so than in low temperature tolerance), but also highlight the fact that relatively small increases in exposure time can impact on mortality.

Information from this study on the heat tolerance of *N. lugens* provides a basis for comparison with temperatures likely to be encountered across different areas of its distribution, but an important question that arises is the extent to which laboratory-derived indices of thermal tolerance can accurately predict survival or mortality under field conditions. The average ‘hot season’ temperatures in tropical lowlands where outbreaks of *N. lugens* occur range from: 20-31°C in India, 25-35°C in Thailand, 26-36°C in Burma, 25-27°C in Indonesia, 22-32°C in Bangladesh, 32-35°C in the Philippines, 20-33°C in Vietnam, 22-27°C in China, 21-24°C in South Korea and 29.9-34.7°C in Malaysia (Mazur, 2011). Whilst these temperatures are generally lower than the CT$_{max}$, HCT and ULT of *N. lugens*, a number of factors will affect survival at high temperature in these climatic areas. Firstly, there will be occasional ‘peak’ temperatures that will pose a greater threat to such tropical insects e.g. 47.2°C in Burma (a record ‘high’ for South-east Asia as a whole) and 49°C in Pakistan (Giese, 2011). Secondly, the CT$_{max}$, HCT and ULT values were estimated from very brief exposures of a few minutes, whereas in nature, high temperatures would be
experienced for much longer periods of time, almost certainly lowering critical tolerance limits below the laboratory-measured values. Also, through climate warming, tropical insects are likely to experience higher temperatures in the future. For example, the mean annual temperature is increasing by 0.23°–1°C per decade in East Asia (China, Japan and South Korea), 0.025°–0.68°C in South-east Asia (Albritton et al., 2002) and 0.26°C in tropical rain forests (Malhi and Wright, 2004). Collectively these data suggest that *N. lugens* is already living close to its upper thermal limit across parts of its distribution.

Apart from lethal effects, the impact of high temperature on mobility, which would affect annual migratory behaviour, is a further limiting factor; and all of these effects are likely to become more detrimental to *N. lugens* and other tropical insects in a warmer climate. There are though further considerations, including intraspecific variation in thermal tolerance related to geographic origin and acclimation ability. The sample population of *N. lugens* used in this study was collected at Pulau Pinang in Malaysia where the annual mean temperature is approximately 27.5°C and minimum and maximum temperatures in the area varied from 23.3 – 24.5°C and 31.3 - 32.8°C respectively over a 15 year period (data from Butterworth Station, Department of Meteorology, Malaysia for 1995 to 2009). Whilst the culture of *N. lugens* was maintained at 23 ± 0.5°C, 16:8 L:D, close to the annual mean temperature for the collection site (see Methods for further details) it is known that acclimation can modify thermal tolerance and critical limits (Fry, 1958; Buffington, 1969; Huey and Bennett, 1990; Sinclair and Roberts, 2005; Terblanche and Chown, 2006; Overgaard et al., 2008; Bale and Hayward, 2010); rearing *N. lugens* at higher temperatures may therefore raise the CT$_{max}$, HCT and ULT values reported here.
In summary, with knowledge of the current mean and occasional peak high temperatures in different parts of the distribution on *N. lugens* and the thermal limits of different life cycle stages, these data in combination provide a basis by which to identify regions within the Asian rice growing area where the insect is likely to become more or less important through future changes in climate; though temperatures may become locally too stressful in some areas, affecting development, reproduction and survival, higher temperatures in other parts of the distribution may allow year-round residency where this is currently impossible. Overall, the pest status of *N. lugens* may not be reduced, but its impact on regional rice production may change over time.

The data presented in this chapter have been published on:

CHAPTER 5

Effects of Acclimation on the Thermal Tolerance of

the Brown Planthopper *Nilaparvata lugens*

5.1 Abstract

The influence of acclimation on the cold and heat tolerance of *Nilaparvata lugens* was determined by measurements of the critical thermal minimum and maximum (CT<sub>min</sub> and CT<sub>max</sub>), chill and heat coma temperature (CCT and HCT) and lower and upper lethal temperature (LLT<sub>50</sub> and ULT<sub>50</sub>). First instar nymphs were acclimated for 5 days at 15°C and for 2 days at 30°C and compared with a population maintained at 23°C; for the adult comparisons, first instar nymphs were reared at 15, 23 and 30°C until adult emergence, requiring development periods of 50-55, 30-35 and 18-20 days respectively.

The thermal tolerance limits of both age groups changed significantly with acclimation and were correlated with rearing temperature. The CT<sub>min</sub> of nymphs reared or acclimated at 15, 23 and 30°C were 12.5 ± 0.3, 15.3 ± 0.3 and 17.6 ± 0.7°C respectively; the equivalent values for adult females were 8.1 ± 0.2, 13.1 ± 0.4 and 16.4 ± 0.9 and 8.8 ± 0.2, 12.9 ± 0.4 and 16.4 ± 0.9 for males. The CT<sub>max</sub> values at the three temperatures were 34.2 ± 0.2, 34.9 ± 1.3 and 37.2 ± 0.1 (first instar nymphs), 36.0 ± 0.1, 37.0 ± 1.0 and 37.3 ± 0.1 (adult female) and 36.6 ± 0.1, 37.4 ± 0.7 and 37.7 ± 0.1°C (adult male). The LLT<sub>50</sub> for the 15, 23 and 30°C populations were 0.5 ± 0.4, 2.3 ± 0.4 and 3.6 ± 0.8 for nymphs and -2.7 ± 0.4, 0.9 ± 0.5 and 2.1 ± 0.3°C for adults; the
equivalent $\text{ULT}_{50}$ values were 40.8 ± 0.4, 41.8 ± 0.1 and 42.9 ± 0.1 for nymphs, and 42.1 ± 0.3, 42.5 ± 0.1 and 43.6 ± 0.1°C for adults.

Across the 48 separate measurements of thermal tolerance ($\text{CT}_{\text{min}}$, CCT, $\text{CT}_{\text{max}}$, HCT, LLT$_{50}$ and $\text{ULT}_{50}$ of nymphs and adult males and females), the temperature difference in comparison with the 23°C reared population was greater in 12 indices in the samples acclimated at 15°C and in 4 when acclimated at 30°C. In a comparison of the acclimation responses between nymphs and adults reared at 23°C and acclimated at either 15 or 30°C, the temperature differential was greater for adults in 14 of the 20 indices. These data indicate that under the acclimation regimes applied to $N. \text{lugens}$ increases in cold tolerance were greater than heat tolerance, and that acclimation over a generation compared with a single life stage increases tolerance across the thermal spectrum.

### 5.2 Introduction

Insects are susceptible to changes in temperature and water availability because they have relatively large surface area-to-volume ratio (Wharton, 2002; Chown and Nicolson, 2004). Many insects, however, live in habitats that buffer exposure to environmental changes (Schowalter, 2006). Most insects are subject to environmental variability including periods of potentially lethal or stressful abiotic conditions (Nyamukondiwa and Terblanche, 2010). For this reason, maintaining optimal body temperature (Tb), water content, and chemical processes is a challenge in variable environments (Angilletta Jr. et al., 2004). Bullock (1955) reported that many poikilothermic animals exhibit, in their metabolism or activity, some degree of independence
from the ambient temperature. Indeed, insects possess a variety of physiological and behavioural mechanisms that aid survival in variable environments (Bakken, 1976b; Armitage and Stinson, 1980). Adaptive physiological responses can mitigate against exposure to sub-optimal conditions, for example, by acclimatizing to different thermal conditions, and this in turn can impact on performance (Bale et al., 2002; Berger et al., 2011). Nevertheless, insects are often killed by sudden or unexpected changes in the temperature, moisture, or chemical conditions of their habitat (Schowalter, 2006). The influence of temperature on the survival of organisms has received considerable attention (Newell et al., 1971; Addo-Bediako et al., 2000; Berrigan, 2000; Cerdá, 2001; Castañeda et al., 2004; Bickford et al., 2010; Berger et al., 2011). The ability of insects to cope with thermal stress is achieved in one of two ways - thermoregulation of body temperature through behavioural adaptation, and physiological and biochemical mechanisms (Casey, 1992; Feder et al., 1997; Feder and Hoffmann, 1999; Carrascal et al., 2001; Addo-Bediako et al., 2002; Chown and Nicolson, 2004; Cadena and Tattersall, 2009; Angilletta Jr. et al., 2010).

Insects can modify their thermal sensitivity through short-term processes such as acclimatization and long-term processes such as evolutionary adaptation (Huey and Stevenson, 1979; Huey et al., 1999). Thermal acclimation (in the laboratory) or acclimatization (in nature where many environmental factors change) forms part of the range of insect responses to their environment known as phenotypic plasticity that can occur only within limits imposed by the genotype (Prosser and Nelson, 1981; Gibbs et al., 1998). These acclimation responses may involve changes in physiological rate-based processes and performance, thermal niche limits and behaviour, to cope with environmental temperature variation (Bennett and Lenski, 1997; Addo-Bediako et al.,
2002). Behaviourally-based changes represent a more flexible response to environmental variation than physiological processes because animals can respond actively to sensory information to avoid or mitigate lethal conditions (Schowalter, 2006); this is particularly true for highly mobile insects, though limited mobility is not necessarily detrimental in environments with gradients. Additionally, mobile insects may be more able to escape locally stressful conditions and also detect and colonize suitable patches within variable environments.

Animals can acclimatize to seasonal changes of temperature over extended periods of time. For example, many ectotherms become cold-hardened as winter approaches and heat-resistant during the summer. Al-Marzouk (1991) indicated that many poikilothermic species show complete or partial compensation in metabolic rate and movement following acclimation to different temperatures. Whilst the effects of acclimation on insect thermal performance are poorly understood, such information is important for understanding responses to future climate changes and the evolution of these reaction norms (Lachenicht et al., 2010). A number of studies have investigated responses to temperature acclimation in a range of poikilotherms (Aleksiuk, 1971; Newell et al., 1971; Bradley, 1978; Armitage and Stinson, 1980; Al-Marzouk, 1991; Chen et al., 2001; Yalcin et al., 2008). Within the insects a similar relationship between environmental temperature and thermal tolerance has been found across many species whereby the thermal threshold (e.g. lethality) depends on the temperature and the duration of exposure e.g. *Culex pipiens pipiens* (Buffington, 1969), *Periplaneta americana* (Piccione and Baust, 1977), *Sitophilus granaries* L. and *Oryzaephilus surinamensis* L. (Mignon et al., 1996), *Sitobion avenae* (Powell and Bale, 2005), *Locusta migratoria* L.(Wang et al., 2006), *Drosophila melanogaster* (Frazier et al., 2008; Overgaard et al., 2008), *Acheta domesticus* L. (Lachenicht et al., 2010),
Ceratitis capitata and C. rosa (Nyamukondiwa and Terblanche, 2010), Cydia pomonella (Chidawanyika and Terblanche, 2011) and Myzus persicae (Alford et al., 2011).

The brown planthopper, *N. lugens*, is one of the most important rice pests because of its sap-feeding habit and ability to transmit plant-pathogenic viruses (Nakashima et al., 1996). The species is widely distributed in south and east Asia, northern Australia and western Oceania (Claridge et al., 1985; Kawanguchi et al., 2001; Liu and Han, 2006) and makes wind-assisted migratory flights each year to colonize the summer rice growing areas of China, Japan and South Korea (Rosenberg and Magor, 1983). Recent studies on *N. lugens* showed that the upper lethal temperatures of nymphs and adults overlapped with summer high temperatures across parts of its Asian distribution and that insects could be immobilized by heat stress at lower temperatures that would be commonly experienced in some countries every year (Piyaphongkul et al., 2012a; see chapter 4). In a scenario of climate warming it was hypothesized that whereas in some parts of the current distribution higher temperatures might become physiologically limiting, there were other countries where a more favourable future climate would allow year-round survival that is currently prevented by low winter temperatures. As such, the status of *N. lugens* as the most serious pest of rice throughout India and south-east Asia would not necessarily change, but its relative importance might vary over time across its distribution. A subsequent study (Piyaphongkul et al., 2012b; see chapter 6) found that after exposure of nymphs and adults of *N. lugens* at their respective ULT50 (high temperatures that kill 50% of the population), development through the nymphal instars was impeded and fecundity reduced; heat stress affected reproductive fitness in both male and female insects as indicated by reciprocal mating with the untreated gender. These experiments with *N. lugens* were conducted with insects cultured at
23°C, a representative mean temperature for its current distribution. It seems likely, however, that the thermal thresholds determined at this ‘standard’ temperature would differ if populations were maintained at higher or lower temperatures, and detection of any such acclimatory ability will inform predictions on the future status and distribution of this major economic pest (Baker et al., 2000).

The experiments described in this chapter measured three thermal indices (critical thermal minimum and maximum, coma and lethal temperatures) in first instar nymphs and adults of *N. lugens* at both acclimated low and high temperatures in populations of 15° and 30°C and compared these values with data for a ‘control’ population maintained in continuous culture at 23°C. The acclimation treatments applied in this study were not intended to produce ‘fully acclimatized’ insects as would occur under natural environmental conditions. Rather, the aim was to determine whether there was any acclimatory ability in *N. lugens*, to compare responses in insects acclimated in one life cycle stage (first instar nymph) and through a full generation, and to assess the relative changes in the thermal thresholds in response to acclimation at temperatures above and below a standard regime.

### 5.3 Material and methods

#### 5.3.1 Insect materials

The population of *N. lugens* was provided by the MARDI Research Station at Pulau Pinang, Malaysia from insects collected from the field in 2010. The generation time of *N. lugens* at 23°C
is 7 weeks, thus the experimental population had completed 11-12 generations prior to the experiments reported in this study. Rearing conditions have been described previously by Piyaphongkul et al. (2012a; see chapter 4). Briefly, *N. lugens* were reared on *Oryza sativa* L. cv. TN 1 at the maximum tillering stage in individually sealed containers in a quarantine room at 23 ± 0.5°C, 16:8 L:D cycle. For acclimation studies, specimens were cooled or heated from 23°C at 0.1°C min⁻¹ to 15 and 30°C after which they were transferred to temperature-controlled incubators under a 16:8 L:D cycle and maintained at 15 and 30°C. A preliminary study was carried out in which 20 virgin adult females and males were reared as individual pairs on rice plants in perspex boxes at 23°C, 16:8 L:D for 1, 2 and 4 days and then transferred to 15, 23 and 30°C. Each container was checked daily to record the date of egg hatch as indicated by the emergence of first instar nymphs. The aim of this experiment was to determine the time required for mating to occur and to check that eggs could develop and nymphs emerge at the two acclimation temperatures. At 15°C there was no nymph emergence after 25 days and few nymphs had emerged after 35 days. To overcome this problem, nymphs were allowed to emerge from eggs that had developed at 23°C and were then transferred to 15°C. For the 30°C treatment, males and females were kept at 23°C for 4 days (mating period) and then transferred to the higher temperature. The nymphs were held at 15°C for 5 days and at 23 and 30°C for 2 days prior to exposure in the experiments. These acclimation periods reflected the times taken (to the nearest day) at which it could be ensured that individuals were still in the first instar when used in experiments. At 15, 23 and 30°C the insects moulted to adult after 50-55, 35-40 and 18-20 days post-egg hatch respectively. The adults were therefore in the same, directly comparable stage of development in all experiments (Piersma and Drent, 2003; Frazier et al., 2008). During rearing and acclimation through the nymphal instars at the three temperatures *N. lugens* were kept in
plastic containers on rice seedlings. Males and females could be identified at the late fifth instar stage and were separated at this time to prevent mating.

5.3.2 Critical thermal minimum, maximum and coma temperatures

Insect locomotor activity is controlled by the nervous system and consists of coordinated activity that allows selection of environments with favourable conditions, including temperature (Prosser and Nelson, 1981). Thus, measurement of activity thresholds provides an insight into the thermal limits of *N. lugens*. The critical thermal minimum (*CT* min), maximum (*CT* max), chill coma temperature (CCT) and heat coma temperature (HCT) were measured using the system developed by Hazell et al. (2008), comprising an arena within an aluminium block attached to a circulating alcohol bath.

The initial temperature within the arena was set at the rearing or acclimation temperature (15, 23 and 30°C). A sample of 10 first-instar nymphs, adult females or males was placed in the arena and allowed to settle for 15 min after which the temperature was decreased to 10°C or increased to 35°C at 0.5°C min \(^{-1}\). Thereafter, the temperature within the arena was decreased from 10 to -10°C or increased from 35 to 55°C at 0.1°C min \(^{-1}\); these cooling and heating rates were selected to minimise any ‘cold or heat hardening’ response during the change in temperature (Hazell et al., 2010a). The movement of *N. lugens* was recorded using a digital video camera (Infinity 1-1; Lumenera Scientific, Canada) with a macro lens (Computar MLH-10X, CBC Corp., New York, NY) positioned over the arena and linked to a desktop computer. Data on insect movement and temperature were recorded simultaneously by video capture software (Studio Capture DT; Studio 86 Designs, UK). The *CT* min and max were the temperatures at which the insect ceased coordinated...
movement and became immobile; the CCT and HCT were the temperatures at which the last movement of an appendage (antenna, leg) occurred. Each experiment was repeat with a further sample of 10 individuals of each life cycle stage (n = 20).

5.3.3 Lethal temperatures

For the lower lethal (LLT) and upper lethal temperature (ULT) experiments, 10 first-instar nymphs or adults were placed in a 0.9 ml Eppendorf tube (with five replicates of 10 specimens at each exposure temperature), and then placed at the bottom of a glass test tube suspended in a programmable alcohol bath (Haake Phoenix 11 P2; Thermo Electron Corp., Germany with temperature accuracy of ± 0.5°C). The samples were held at their respective rearing or acclimation temperatures (15, 20 or 30°C) for 30 min to reduce the stress associated with handling and then cooled or heated to a range of temperatures at 0.5°C min⁻¹. When the temperature in the alcohol bath reached the target temperature, the insects were held at this temperature for the required period of time to ensure that the entire sample experienced the exposure temperature; preliminary experiments indicated that 2 and 6 min respectively were required for nymphs and adults to reach thermal equilibrium with the set temperature. Thereafter, all samples were heated or cooled back to their rearing temperature at 0.5°C min⁻¹ and then transferred to recovery trays (transparent plastic boxes, 16 x 8.5 x 28 cm³ with 1.22 mm ventilation mesh) containing rice plants at 23°C, 16:8 L:D. Mortality was assessed 72 h after exposure. The data were analyzed by Probit in Minitab 15 (Minitab Inc., 2007) to estimate the temperature at which 50% of the sample of was killed (the LLT₅₀ and ULT₅₀). Handling controls revealed no bias between treatments with 99% survival.
An analysis of variance (ANOVA) was used to test for differences in CT$_{\text{min}}$, CT$_{\text{max}}$, HCT, CCT, ULT$_{50}$ and LLT$_{50}$ between life cycle stages within the same rearing temperature. Where significant differences occurred, the data were further analysed by Tukey's honest significance difference post-hoc test to separate statistically heterogenous groups. A split plot method was used to analyse the interaction between temperature and life cycle stage. All analyses were conducted using SPSS statistical software.

5.4 Results

5.4.1 Critical thermal minimum, maximum and coma temperatures

The effect of acclimation on the critical minimum and chill coma temperatures of *N. lugens* is shown in Table 5.1. Within each acclimation temperature, the results indicated that the adult stages had significantly lower CT$_{\text{min}}$ than nymphs at 15 (F$_{2, 57} = 135.97$, p < 0.001) and 23$^\circ$C (F$_{2, 57} = 13.99$, p < 0.001), but there was no difference at 30$^\circ$C. Additionally, CT$_{\text{min}}$ did not differ between the sexes at 23$^\circ$C. Likewise, the CCTs of adults were significantly lower than for nymphs at 15 (F$_{2, 57} = 323.85$, p < 0.001), 23 (F$_{2, 57} = 147.39$, p < 0.001) and 30$^\circ$C (F$_{2, 57} = 180.50$, p < 0.001). The CCT also differed significantly between the sexes at 15 and 30$^\circ$C, but not at 23$^\circ$C. The changes in mean CT$_{\text{min}}$ of first instar nymphs, adult females and adult males across the three temperatures were significant (F$_{2, 114} = 125.39$, p < 0.01). There was however, no difference in mean CT$_{\text{min}}$ among nymphs, adult females and adult males (F$_{2, 114} =0.12$, p=0.727), nor between the interaction of temperature treatment and life cycle stage. By comparison, there was a significant effect of rearing temperature on CCT (F$_{2, 114} = 200.91$, p < 0.01), with a
difference between life cycle stages ($F_{2, 114} = 16.17, p < 0.01$) but not in the interaction between treatment and life cycle stage ($F_{2, 114} = 0.79, p = 0.456$).

**Table 5.1** Mean $CT_{\text{min}}$ and chill coma temperature (CCT) ± SE of *N. lugens* acclimated to 15, 23 and $30^\circ$C (n= 20 for each life stage and sex).

<table>
<thead>
<tr>
<th>Index</th>
<th>Rearing temperature ($^\circ$C)</th>
<th>Mean temperature ($^\circ$C)</th>
<th>F value, p (Two way ANOVA)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nymphs</td>
<td>Adult female</td>
</tr>
<tr>
<td>$CT_{\text{min}}$</td>
<td>15</td>
<td>12.5±0.3</td>
<td>8.1±0.2</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>15.3±0.3</td>
<td>13.1±0.4</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>17.6±0.7</td>
<td>16.4±0.9</td>
</tr>
<tr>
<td>CCT</td>
<td>15</td>
<td>6.2±0.2</td>
<td>-1.2±0.3</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>6.7±0.2</td>
<td>2.1±0.2</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>6.8±0.1</td>
<td>2.9±0.2</td>
</tr>
</tbody>
</table>

Acclimation at $15^\circ$C reduced both the $CT_{\text{min}}$ and CCT in *N. lugens* (Figures 5.1 and 5.2). There was a significant difference in $CT_{\text{min}}$ between acclimation temperatures for nymphs ($F_{2, 57} = 28.69, p < 0.001$), adult females ($F_{2, 57} = 54.77, p < 0.001$) and adult males ($F_{2, 57} = 77.01, p < 0.001$) (Figure 5.1). Similarly, populations acclimated to $15^\circ$C had a lower CCT than those reared at 23 or acclimated at $30^\circ$C (Figure 5.2). The CCT differed significantly between acclimation
temperatures for nymphs ($F_{2, 57} = 3.37, p = 0.041$), adult females ($F_{2, 57} = 101.44, p < 0.001$) and adult males ($F_{2, 57} = 99.23, p < 0.001$). However, the CCT of first instar nymphs acclimated at $15^\circ C$ was similar to those maintained at $23^\circ C$.

\[ \text{Figure 5.1 } \text{CT}_{\text{min}} \text{ of } N. \text{lugens acclimated at } 15^\circ C \text{ (white bars), } 23^\circ C \text{ (black bars) and } 30^\circ C \text{ (cross-hatch bars. Mean } \text{CT}_{\text{min}} \text{ of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter within each graph frame are not significantly different (p } \leq 0.05) \text{; } n = 20 \text{ for each life cycle stage.} \]
Figure 5.2 Chill coma temperature (CCT) of *N. lugens* acclimated at 15 (white bars), 23 (black bars) and 30°C (cross-hatch bars). Mean CCT of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter are not significantly different (*p* ≤ 0.05); *n* = 20 for each life cycle stage.
The effect of acclimation on the critical maximum and heat coma temperatures of *N. lugens* is summarised in Table 5.2. The CT$_{\text{max}}$ was significantly lower in first instar nymphs than adults at 15 ($F_{2, 57} = 59.77, p < 0.001$), 23 ($F_{2, 57} = 33.55, p < 0.001$) and 30$^\circ$C ($F_{2, 57} = 7.84, p < 0.001$). There was no difference between the sexes at 23$^\circ$C nor between nymphs and adult females at 30$^\circ$C. In the same way, the adult stage had a significantly higher HCT than nymphs at 15 ($F_{2, 57} = 89.47, p < 0.001$), 23 ($F_{2, 57} = 68.21, p < 0.001$) and 30$^\circ$C ($F_{2, 57} = 33.96, p < 0.001$). HCT also differed significantly between the sexes and was higher in females at all acclimation temperatures. No individuals survived exposure at the HCT.

Across the three temperatures there was a significant effect of rearing temperature ($F_{2, 114} = 39.11, p < 0.001$), with a difference between life cycle stages ($F_{2, 114} = 20.35, p < 0.001$), but not in the interaction between rearing temperature and life cycle stage ($F_{2, 114} = 0.51, p = 0.601$). Similarly, changes in rearing temperatures had a significant effect on mean HCT ($F_{2, 114} = 43.02, p < 0.01$), with difference between life cycle stages ($F_{2, 114} = 29.09, p < 0.01$), but not in the interaction between rearing temperature and life cycle stage ($F_{2, 114} = 0.96, p = 0.386$).
Table 5.2 Mean $\text{CT}_{\text{max}}$ and heat coma temperature (HCT) ± SE of $N. \text{lugens}$ acclimated to 15, 23 and 30°C (n= 20 for each life stage and sex).

<table>
<thead>
<tr>
<th>Index</th>
<th>Rearing temperature ($^\circ$C)</th>
<th>Mean temperature ($^\circ$C)</th>
<th>F value, p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nymph</td>
<td>Adult female</td>
</tr>
<tr>
<td>$\text{CT}_{\text{max}}$</td>
<td>15</td>
<td>34.2±0.2</td>
<td>36.0±0.1</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>34.9±1.3</td>
<td>37.0±1.0</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>37.2±0.1</td>
<td>37.3±0.1</td>
</tr>
<tr>
<td>HCT</td>
<td>15</td>
<td>37.6±0.1</td>
<td>41.6±0.3</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>37.7±1.3</td>
<td>43.4±1.7</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>41.0±0.3</td>
<td>45.0±0.1</td>
</tr>
</tbody>
</table>

The $\text{CT}_{\text{max}}$ and HCT of all life stages examined were highest in populations acclimated at 30 and lowest in those acclimated at 15°C (Figures 5.3 and 5.4). There were significant differences in $\text{CT}_{\text{max}}$ between acclimation temperatures for nymphs ($F_{2, 57} = 47.88, p < 0.001$), adult females ($F_{2, 57} = 18.91, p < 0.001$) and adult males ($F_{2, 57} = 21.38, p < 0.001$) (Figure 5.3). Similarly, $N. \text{lugens}$ acclimated to 30°C had a higher HCT than those reared at 23°C or acclimated at 15°C (Figure 5.4). HCT differed significantly between temperatures for nymphs ($F_{2, 57} = 89.47, p < 0.001$), adult females ($F_{2, 57} = 68.21, p < 0.001$) and adult males ($F_{2, 57} = 33.96, p < 0.001$).
Figure 5.3 CT\textsubscript{max} of \textit{N. lugens} acclimated at 15 (white bars), 23 (black bars) and 30°C (cross-hatch bars). Mean CT\textsubscript{max} of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter are not significantly different (p ≤ 0.05); n = 20 for each life cycle stage.
Figure 5.4 Heat coma temperature (HCT) of *N. lugens* acclimated at 15 (white bars), 23 (black bars) and 30°C (cross-hatch bars. Mean HCT of A) first instar nymph B) adult females and C) adult males. Mean values with the same letter are not significantly different (*p* ≤ 0.05); *n* = 20 for each life cycle stage.
5.4.2 Lethal temperatures

The LLT$_{50}$ and ULT$_{50}$ of *N. lugens* acclimated to 15, 23 and 30°C are summarized in Table 5.3. Within each acclimation temperature adults had significantly lower LLT$_{50}$ than first instar nymphs at 15°, $F_{1, 8} = 47.21$, $p < 0.001$, 23°, $F_{1, 8} = 6.92$, $p = 0.030$ and at 30°C, $F_{1, 8} = 11.73$, $p = 0.009$. Also, within the three acclimation temperatures, the mean (± SE) ULT$_{50}$ of adults was significantly higher than for first instar nymphs at 15°, $F_{1, 8} = 7.49$, $p = 0.026$, 23°, $F_{1, 8} = 17.52$, $p = 0.003$ and at 30°C, $F_{1, 8} = 45.58$, $p < 0.000$.

Across the three temperatures, the LLT$_{50}$ was lower in both nymphs and adults at 15°, 23° and 30°C respectively and the ULT$_{50}$ correspondingly higher when acclimated at the higher temperatures. From two way ANOVA analyses, there was a significant effect of rearing temperature on mean LLT$_{50}$ ($F_{2, 24} = 37.45$, $p < 0.001$), with a difference between life cycle stages ($F_{2, 24} = 49.81$, $p < 0.001$), but no difference in the interaction between the treatment and life cycle stage ($F_{2, 24} = 2.57$, $p = 0.098$). By comparison, the changes in mean ULT$_{50}$ across the three temperatures was significant ($F_{2, 24} = 35.76$, $p < 0.001$), with a difference between the life stages ($F_{2, 24} = 11.39$, $p = 0.003$) and in the interaction between the temperature treatment and life stage ($F_{2, 24} = 6.54$, $p = 0.005$).

The LLT$_{50}$ of *N. lugens* decreased with an increasing rearing temperature (Figure 5.5). The LLT$_{50}$ differed significantly between the three acclimating temperatures in both nymphs ($F_{2, 12} = 10.41$, $p = 0.002$) and adults ($F_{2, 12} = 37.78$, $p < 0.001$) (Figure 5.5A and 5.5B). However, within the same life cycle stage, the LLT$_{50}$ of nymphs acclimated to 15°C and the 23°C population were similar, as were adults acclimated to 30°C and those reared at 23°C.
An increase in acclimation temperature increased the \( \text{ULT}_{50} \) of *N. lugens* in both life cycle stages and sexes. The \( \text{ULT}_{50} \) was significantly different between acclimation temperatures in both nymphs (\( F_{2, 12} = 22.82, p < 0.001 \)) and adults (\( F_{2, 12} = 18.45, p < 0.001 \)) (Figure 5.6A and 5.6B). However, no differences were found in the \( \text{ULT}_{50} \) of first instar nymphs reared at 23 and 30\(^\circ\)C, nor between adults reared at 15 and 23\(^\circ\)C.

**Table 5.3** \( \text{LLT}_{50} \) and \( \text{ULT}_{50} \) of nymphs and adults of *N. lugens* at 15\(^\circ\), 23\(^\circ\) and 30\(^\circ\)C.

<table>
<thead>
<tr>
<th>Index</th>
<th>Rearing temperature (( ^\circ)C)</th>
<th>Mean temperature (( ^\circ)C)</th>
<th>F value, p</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Nymphs</td>
<td>Adults</td>
</tr>
<tr>
<td><strong>LLT(_{50})</strong></td>
<td>15</td>
<td>0.5 ±0.4</td>
<td>2.7±0.4</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>2.3±0.4</td>
<td>0.9±0.5</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>3.6±0.8</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td><strong>ULT(_{50})</strong></td>
<td>15</td>
<td>40.8±0.4</td>
<td>42.1±0.3</td>
</tr>
<tr>
<td></td>
<td>23</td>
<td>41.8±0.1</td>
<td>42.5±0.1</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>42.9±0.1</td>
<td>43.6±0.1</td>
</tr>
</tbody>
</table>
Figure 5.5 Lower lethal temperature (LLT_{50}) of first instar nymphs and adults of *N. lugens* acclimated to 15\(^\circ\) (white bars), 23\(^\circ\) (black bars) and 30\(^\circ\) (cross-hatch bars). Mean LLT_{50} of A) first instar nymph and B) Adults. Mean values with the same letter are not significantly different (p ≤ 0.05); n = 50 at each exposure temperature.
Figure 5.6 Upper lethal temperature ($\text{ULT}_{50}$) of first instar nymphs and adults of *N. lugens* acclimated to $15^\circ$ (white bars), $23^\circ$ (black bars) and $30^\circ$C (cross-hatch bars). Mean $\text{ULT}_{50}$ of A) first instar nymph and B) Adults. Mean values with the same letter are not significantly different ($p \leq 0.05$); $n = 50$ at each exposure temperature.
5.4.3 Low and high temperature changes in thermal thresholds

A total of 48 independent measurements of the thermal thresholds of *N. lugens* were made across the three temperatures (Tables 5.1, 5.2 and 5.3). In comparison with the population in culture at 23°C, acclimation at 15°C consistently lowered the measured values, and acclimation at 30°C resulted in corresponding increases (Table 5.4).

The temperature difference in comparison with the 23°C reared population was greater in 12 indices in samples acclimated at 15°C and in 4 when acclimated at 30°C. In a comparison of the acclimation responses between nymphs and adults reared at 23°C and acclimated at either 15 or 30°C, the temperature differential was greater for adults in 14 of the 20 indices. These data indicate that under the acclimation regimes applied to *N. lugens* most increases in cold tolerance were greater than heat tolerance, and that acclimation over a generation compared with a single life stage was more likely to increase tolerance across the thermal spectrum.
Table 5.4 Temperature differential (°C) in thermal thresholds of *N. lugens* after acclimation at 15° and 30°C in comparison with a population maintained at 23°C.

<table>
<thead>
<tr>
<th>Thermal indicator</th>
<th>15°C</th>
<th>30°C</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Nymphs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CT</em>&lt;sub&gt;min&lt;/sub&gt;</td>
<td>2.8</td>
<td>2.3</td>
</tr>
<tr>
<td>Chill coma</td>
<td>0.5</td>
<td>0.1</td>
</tr>
<tr>
<td><em>CT</em>&lt;sub&gt;max&lt;/sub&gt;</td>
<td>0.7</td>
<td>2.3</td>
</tr>
<tr>
<td>HCT</td>
<td>0.1</td>
<td>3.3</td>
</tr>
<tr>
<td><strong>Adult females</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CT</em>&lt;sub&gt;min&lt;/sub&gt;</td>
<td>5.0</td>
<td>3.3</td>
</tr>
<tr>
<td>Chill coma</td>
<td>3.3</td>
<td>0.8</td>
</tr>
<tr>
<td><em>CT</em>&lt;sub&gt;max&lt;/sub&gt;</td>
<td>1.0</td>
<td>0.3</td>
</tr>
<tr>
<td>HCT</td>
<td>1.8</td>
<td>1.6</td>
</tr>
<tr>
<td><strong>Adult males</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>CT</em>&lt;sub&gt;min&lt;/sub&gt;</td>
<td>4.1</td>
<td>3.5</td>
</tr>
<tr>
<td>Chill coma</td>
<td>2.9</td>
<td>0.9</td>
</tr>
<tr>
<td><em>CT</em>&lt;sub&gt;max&lt;/sub&gt;</td>
<td>0.8</td>
<td>0.3</td>
</tr>
<tr>
<td>HCT</td>
<td>1.4</td>
<td>1.2</td>
</tr>
<tr>
<td><strong>Nymphs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>LLT</em>&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.8</td>
<td>1.3</td>
</tr>
<tr>
<td><em>ULT</em>&lt;sub&gt;50&lt;/sub&gt;</td>
<td>1.0</td>
<td>1.1</td>
</tr>
<tr>
<td><strong>Adults</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>LLT</em>&lt;sub&gt;50&lt;/sub&gt;</td>
<td>3.6</td>
<td>1.2</td>
</tr>
<tr>
<td><em>ULT</em>&lt;sub&gt;50&lt;/sub&gt;</td>
<td>0.4</td>
<td>1.1</td>
</tr>
</tbody>
</table>
5.5 Discussion and conclusion

Temperature can have a marked effect on physiological processes (Terblanche et al., 2008). Many ectotherms live in environments which experience daily or seasonally fluctuating temperatures (Armitage and Stinson, 1980), and in some regions of the world, these short term variations occur within an era of longer term global climate change. The ability to cope or respond to changes in temperature can have a major impact on survival (Bradley, 1978; Rinehart et al., 2007). The general conclusion to emerge from this study is that *N. lugens*, a species with a wide distribution in tropical and sub-tropical areas, is able to acclimate to temperatures that are both lower and higher than its rearing temperature, with corresponding decreases and increases in thermal thresholds, including its lethal temperatures. It has been suggested that many poikilothermic species show complete or partial compensation in metabolic rate and movement following acclimation in either low or high temperature regimes because of changes in the enzyme activity in muscle cells (Al-Marzouk, 1991, Chen et al., 2001).

At extremes of cold and heat, many insects respond by a loss of motor coordination and equilibrium that are reversible, and occur with smaller changes in temperature than are required to inactivate enzymes. Development at high temperatures and associated acclimatory responses may allow *N. lugens* to live in a range of ‘high thermal environments’ and to survive the highest summer temperatures in such areas because their critical temperatures may change adaptively. Moreover, by modifying their temperature tolerance range, *N. lugens* can mitigate the effects of both short term environmental fluctuations and longer term seasonal changes.
When first instar and adult *N. lugens* were acclimated at 15°C, all lower thermal thresholds (*CT_{\text{min}}, CCT* and *LLT}_{50}*) decreased relative to the population maintained at the culture temperature of 23°C, and in turn, these indices were lower at 23° than at 30°C. In a similar pattern, all the higher thresholds (*CT_{\text{max}}, HCT* and *ULT}_{50}*) increased relative to the 23°C culture when acclimated at 30°C, and these upper thermal values were higher at 23° than at 15°C (Tables 5.1-5.3). The processes by which insects can increase their cold tolerance and maintain activity at lower temperatures include the synthesis of cryoprotectants (Wang et al., 2006) and heat shock proteins (Feder and Hofmann, 1999; Sørensen et al., 2003; Rinehart et al., 2007), decrease in body water content, (Gates, 1980), and changes in metabolic rate in colder environments or seasons (Piccione and Baust, 1977). Heat shock proteins function as molecular chaperones during periods of stress, binding to other proteins to minimize the detrimental effects of misfolding and then promoting the return of these proteins to their normal conformations when favorable conditions again prevail (Rinehart et al., 2007).

Increases in cold tolerance after ‘cold acclimation’ have been observed in a number of species. For example, Overgaard et al. (2008) found that in an exposure of *Drosophila melanogaster* for 60 h at 0°C, more than 80% of flies acclimated at 15°C survived but there was 100% mortality of flies that had been maintained at 25°C; the *LLT}_{50} decreased by 1.5°C in the 15°C acclimated flies in comparison with those reared at 25°C. Likewise, the cold tolerance of the fruit flies *Ceratitis capitata* and *C. rosa* cultured at 25°C was increased after acclimation at 20°C for 7 days (Nyamukondiwa and Terblanche, 2010). Also, the rapid cold hardening response first reported in the flesh fly *Sarcophaga crassipalpis* (Lee and Baust, 1985) has since been found to be common in insects across a range of species from different trophic guilds, climatic origin and voltinism.
Powell and Bale (2005) found that rapid cold hardening occurred in nymphs and adults of the grain aphid *Sitobion avenae* that had been cultured at 20°C, and that a further rapid increase in cold hardiness could be induced in populations that had been acclimated at 10°C.

This study also showed that acclimation at 30°C could increase heat tolerance in terms of changes in the ULT$_{50}$, CT$_{\text{max}}$ and HCT. Under conditions of heat stress insects up-regulate heat shock proteins to minimize stress-induced protein aggregation and facilitate removal of damaged protein (Krebs and Feder, 1998; Feder and Hofmann, 1999; Edgerly et al., 2005). The importance of the heat shock protein response for insects has been well reported (Feder and Krebs, 1998; Feder and Hofmann, 1999; Salvucci et al., 2000; Kim et al., 2008). In general, however, the scale of responses at high temperatures is less than after acclimation at a lower temperature as was found in this study. Overgaard et al. (2008) also found that heat tolerance of *D. melanogaster* was not influenced by acclimation at 15, 20 and 25°C, while Lachenicht et al. (2010) reported that heat knockdown resistance of *Acheta domesticus* was more responsive than chill coma recovery time to acclimation at 25, 29 and 33°C. In nature, upper and lower thermal limits both decline with latitude, but the decrease is more pronounced for lower thermal limits (Huey, 2010).

There are a number of reasons that may explain why changes in heat tolerance in response to acclimation occur over a narrower temperature range than changes in cold tolerance. First, there is a difference in physiological problems posed by high and low temperatures in terms of effects on the structural integrity of biomolecules (Edgerly et al., 2005; Gullan and Cranston, 2010). The damage and death caused by high temperature is attributed to protein denaturation, membrane and enzyme structure alteration, and water loss or dehydration (Hazel, 1995; Somero, 1995; Koffler et al., 1957; Kumar et al., 2000; Hance et al., 2007; Dillion et al., 2010). Inherently, the
stability of non-covalent bonds that govern the complex structure of proteins also determines the upper thermal limits and below this threshold there are many different but interrelated temperature-dependent biochemical reactions (Gullan and Cranston, 2010). The potential deleterious effects of low temperature differ from heat stress in a number of ways. In general, extreme low temperature exposure mainly involves a change in the state of water within the organism from liquid to solid (Wharton, 2002). To minimise this problem the insects may possess one or several mechanisms e.g. synthesis of cryoprotectants (and heat shock proteins) that allow survival at such cold extremes (Feder and Hofmann, 1999; Sørensen et al., 2003; Rinehart et al., 2007). Also, the physiological and biochemical problems caused by low temperature seem to be more frequently reversible than with heat stress (Hazell et al., 2008, Hazell et al., 2010a).

Second, insects are vulnerable to fluctuations in environmental temperature because of effects on metabolic rate (Prosser and Nelson, 1981; Hazel, 1995; Krebs and Holbrook, 2001). As temperature decreases, metabolism slows as the kinetic energy imparted to chemical reactions also decreases. Moreover, at thermal extremes, nervous systems are often impaired before other functions and hence behavioural responses may be negatively impacted before protein denaturation occurs (Prosser and Nelson, 1981). Thus, as temperature decreases, insect movement will stop at species-specific values (Hazell et al., 2010a). The temperature at which insects cease movement at low temperature ($CT_{min}$) is however, normally above their lower lethal threshold and does not change protein structure; hence this effect is potentially reversible. By contrast, an increase in temperature within the ‘favourable range’ for development will increase the metabolic rate of the insect (Gullan and Cranston, 2010). As a result, when environmental temperatures increase above the optimum temperature to the $CT_{max}$ and HCT, the effect on
mobility is usually irreversible as these temperatures are at or close to the upper lethal temperature (Piyaphongkul et al., 2012a). In addition, with increases in metabolic rate, the higher consumption of energy or nutrients may compromise other cellular functions (Krebs and Holbrook, 2001).

Last, acclimation to temperature perturbation may be deleterious with respect to fitness and impose costs on the organism (Bennette and Lenski, 1997,) such as changes in cell metabolism that follow a physiological response (Krebs and Holbrook, 2001). For example, high expression of heat shock proteins may alter the specific activity of important enzymes because the defence proteins interfere with enzymatic processes within the cell (Krebs and Loeschcke, 1996; Edgerly et al., 2005). Thus with *N. lugens*, although they are able to synthesise heat shock proteins (Kim et al., 2008), there may be a trade-off in this response with negative impacts on growth and development (Krebs and Feder, 1997) explaining, at least in part, why acclimation to high temperature occurs over a relatively narrow range.

Information acquired from this study on the critical thermal thresholds and acclimatory ability of *N. lugens* can be related to the possible effects of climate warming on the distribution of this species in temperate sub-regions in Asia. Many studies have reported a progressive warming trend for most regions in Asia, which is most pronounced over northern and north-eastern areas e.g. China, Japan, South Korea, Vietnam and India (Wigley et al., 1980; Ramanathan et al., 2007; Kim, 2010). There is the suggestion that long-term summer and winter climate variations in China may be connected to the warming trend in the sea surface temperature of the Indian Ocean and increases in greenhouse gas emissions (Hu et al., 2003). Climate models indicate that increases of 2-3°C are likely over the next century (Wigley et al., 1980) and extreme high
temperature events may occur in temperate regions such as the high temperatures and severe
droughts that occurred in many areas in 1994, including northern China, South Korea and Japan
(Yoo et al., 2004). Comparisons of the acclimation ability of *N. lugens* at 15, 23 and 30°C
demonstrated some ability to survive at low temperature; for example, approximately 50% of
15°C-acclimated nymphs and adults survived at 3.6 and 2.1°C respectively. These levels of cold
tolerance, at least in short term exposures, suggest that there would be some winter survival in
China, Japan and South Korea. Also, in some areas in the Asian temperate sub-region where year
round survival is currently not possible, the combined effects of a warmer climate and cold
acclimation may further aid the survival of *N. lugens* in such areas. Changes in distribution linked
to climate change may have impacts on the agricultural importance of *N. lugens* across its current
distribution. There are however, two important caveats to this conclusion. Firstly, natural winter
exposure will be for longer periods of time than in laboratory experiments used to determine
lethal limits, and secondly, the relatively high threshold temperature at which eggs hatch (15°C)
would limit the development of such populations (Kuno, 1979; Dyck and Thomas, 1979;
Piyaphongkul et al., 2012b). This is the main explanation as to why populations of *N. lugens* in
tropical areas can remain in paddy fields throughout the year, whereas in temperate regions such
as Japan and South Korea, the population is replaced in the summer of each year by immigrants
from more southerly regions (Khush, 1979; Kuno, 1979; Rosenberg and Magor, 1983; Seo et al.,
2009b; Seo et al., 2010).

A further area of interest arising from this study is whether the limited acclimation ability of *N.
lugens* to high temperature will be sufficient protection against extreme heat stress events that
will occur as part of climate warming. Many studies have now provided evidence that south-east
Asia (the area including Burma, Cambodia, Thailand, Laos, peninsular Malaysia, Vietnam, the Philippines, and Indonesia), has warmed by at least 0.3°C over recent decades (Heaney, 1991) and temperatures are projected to increase by 1.1 to 4.5°C by the year 2070 (Peh, 2007). Malhi and Wright (2004) reported that all tropical rainforest regions have experienced warming at the mean rate of 0.26±0.05°C per decade since 1960. Moreover, increasingly frequent extreme weather events have occurred across the Asia-Pacific region over the same time periods (Gong et al., 2004; Griffiths et al., 2005; Francisco, 2008; Choi et al., 2009). Correlations between mean temperature and the frequency of extreme temperatures are strongest in the tropical Pacific Ocean from French Polynesia to Papua New Guinea, Malaysia, the Philippines, Thailand and southern Japan (Griffiths et al., 2005). On the basis of analyses of temperature data from 91 stations in 15 countries in the South Pacific from 1961 to 1998, Manton et al., (2001) reported that the annual number of hot days and warm nights had significantly increased over the time period, with a corresponding significant decrease in the annual number of cool days and cold nights. In general, there is less seasonal change in surface air temperatures in the tropics than in other regions, hence tropical insects are adapted to a relatively uniform and narrow temperature range with the result that at the extremes of the acclimation range, the preferred temperature may not change (Deutsch et al., 2008; Larsen et al., 2011b). This may render tropical species more sensitive and vulnerable to climate change. The data from this study supports this view; thus, although *N. lugens* acclimated at 30°C are more able to survive at extreme high temperatures than populations reared at 15 and 23°C, where natural populations are already living close to their upper thermal limits, the limited acclimation response to high temperature may not be sufficient to protect against irregular extreme events. More generally, increasing temperature and associated heat stress in south-east Asia have the potential to modify the abundance and distribution of *N. lugens* as
ectothermic organisms perform increasingly sub-optimally at the high end of their thermal tolerance (Bickford et al., 2010, Piyaphongkul et al., 2012a). Climate warming and extreme events are also likely to exert negative effects on other tropical species (Corlett and Lafrankie, 1998; Dudgeon, 2000).

In summary, there have been few previous studies that have combined investigation of the physiological, behavioural and ecological responses of insects to temperature, especially with regard to tropical species, related in part to difficulties of characterising the thermal environments occupied by species of interest (Bryant et al., 2002; Edgerly et al., 2005). Information gained from this study on the scale of acclimation responses and impacts on critical thermal thresholds provide a basis for translating such ecophysiological data to natural environments, allowing some integrated modeling of climate, regional population dynamics and pest status.
CHAPTER 6

Effects of Heat Stress on the Development and Fecundity of the Brown Planthopper *Nilaparvata lugens*

6.1 Abstract

This study investigated the effects of sub-lethal high temperatures on the development and reproduction of the brown planthopper *Nilaparvata lugens*. When first instar nymphs were exposed at their ULT$_{50}$ (41.8°C) mean development time to adult was increased in both males and females, from 15.2 ± 0.3 and 18.2 ± 0.3 days respectively in the control to 18.7 ± 0.2 and 19 ± 0.2 days in the treated insects. These differences in development arising from heat stress experienced in the first instar nymph did not persist into the adult stage (adult longevity of 23.5 ± 1.1 and 24.4 ± 1.1 days for treated males and females compared with 25.7 ± 1.0 and 20.6 ± 1.1 days in the control groups), although untreated males lived longer than untreated females. Total mean longevity was increased from 38.8 ± 0.1 to 43.4 ± 1.0 days in treated females, but male longevity was not affected (40.9 ± 0.9 and 42.2 ± 1.1 days respectively).

When male and female first instar nymphs were exposed at their ULT$_{50}$ of 41.8°C and allowed to mate on reaching adult, mean fecundity was reduced from 403.8 ± 13.7 to 128.0 ± 16.6 eggs per female in the treated insects. Following exposure of adult insects at their equivalent ULT$_{50}$ (42.5°C), the three mating combinations of treated male x treated female, treated male x untreated female, and untreated male x treated female produced 169.3 ± 14.7, 249.6 ± 21.3 and 233.4 ± 17.2 eggs per female respectively, all significantly lower than the control (403.8 ± 13.7).
Exposure of nymphs and adults at their respective ULT_{50} temperatures also significantly extended the time required for their progeny to complete egg development for all mating combinations compared with the control. Overall, sub-lethal heat stress inhibited nymphal development, lowered fecundity and extended egg development time.

6.2 Introduction

The effects of climate change on organisms and ecological communities are a highly topical issue. Insects are a taxon with limited ability to regulate their body temperature and are thus directly impacted by both prevailing weather and longer term climate change. Research on insect-climate interactions has focused on the measurement of thermal thresholds and lethal limits (Klok et al., 2004; Renault et al., 2005; Klose et al., 2008; Hanna and Cobb, 2007), responses to manipulated conditions representing different scenarios of climate warming (Estay et al., 2009; Hegland et al., 2009; Bale and Hayward, 2010; Hofmann and Todgham, 2010) and shifts in distributions or changes in phenology detected through analyses of long term datasets (Kersting et al., 1999; Parmesan et al., 1999; Karban and Strauss, 2004; Terblanche and Chown, 2006; Musolin, 2007; Liefting et al., 2010, Nethrer and Schopf, 2010). In general, more is known about the low temperature ecophysiology of insects (Block et al., 1990; Bale et al., 2000; Shreve et al., 2004; Powell and Bale, 2005; Sinclair and Roberts, 2005; Lapointe et al., 2007; Elnitsky et al., 2008; Macmillan and Sinclair, 2011) than the effects of high temperatures, though upper thermal limits have been measured for a number of species (Fischer et al., 2010; Hazell et al., 2010a; Chidawanyika and Terblanche, 2011; Zerebecki and Sorte, 2011; Piyaphongkul et al., 2012a).
Also, whilst many studies have measured critical thermal thresholds at both low (Harrington and Cheng, 1984; Shreve et al., 2004; Iranipour et al., 2010; Hazell and Bale, 2011; Macmillan and Sinclair, 2011) and high temperatures (Woodrow and Grace, 1998; Hallman et al., 2005; Renault et al., 2005; O’Neill and Rolston, 2007; Terblanche et al., 2008; Lalouette et al., 2011), less is known about the impacts of sub-lethal thermal stress on surviving individuals, though effects on development and reproduction have been reported (Okasha, 1968; Okasha, 1970; McDonald et al., 1997; Morgan, 2000; Hance et al., 2007). Climate change can affect terrestrial ectothermic species by modifying the structure of their physical environment, and by the associated changes in the thermal regime or temperature profile of the habitat (Heath et al., 1971; Miles, 1994; Warren et al., 2001). The mechanistic link between the biophysical environment and individual performance will directly affect demographic (e.g. survivorship, growth and reproduction) and population level phenomena (e.g. density and age structure) (Dunham et al., 1989). Thus, a central issue in insect ecophysiology is how environmental factors such as temperature affect physiological performance (Angilletta Jr. et al., 2002; Klok et al., 2004; Kingsolver et al., 2007; Lailvaux and Irschick, 2007; Overgaard et al., 2008). Temperature has a direct effect on the growth and development of insects (Knapp and Casey, 1986; Blanckenhorn, 1997; Mehrparvar and Hatami, 2007; Bowler and Terblanche, 2008; Sanuy et al., 2008; Angilletta Jr. et al., 2010). The temperature-development relationship is approximately linear, increasing progressively to a maximum level beyond which the rate decreases and the response curve becomes markedly asymmetrical through the effects of heat stress and approaching lethality (Huey and Bennett, 1990; Kingsolver and Woods, 1997; Huey and Berrigan, 2001; Folk et al., 2007; Lapointe et al., 2007; Rezink et al., 2009). In addition, both longevity and fecundity of insects reach a maximum at species-specific optimum temperatures and more or less symmetrically decrease at both the
lower and upper limits of tolerance (Irwin and Lee, 2000; Zani et al., 2005). Understanding the behavioural and physiological responses of insects to thermal stress will inform predictions about how climate warming could affect distributions, changes in pest status, and the likelihood of species extinctions (Amarasekare and Savage, 2012). A number of studies have investigated the effects of temperature on development and fecundity e.g. *Nilaparvata lugens* (Hou and Lee, 1984; Chu and Yang, 1985; Lee and Hou, 1987; Noda et al., 1995; Cohen et al., 1997; Krishnaiah et al., 2005; Chen et al., 2011), small brown planthopper *Laodelphax striatellus* (Okasha, 1970; Zhang et al., 2008; Liu and Zhang, 2012), the butterfly *Pararge aegeria* (Berger et al., 2008) and the pea leafminer *Liriomyza huidobrensis* (Huang et al., 2007).

The brown planthopper *Nilaparvata lugens* is the most serious rice pest in Asia, affecting a wide range of economically important rice crops that arose from the green revolution (Sōgawa, 1982; Saxena and Barrion, 1983; Visarto et al., 2006; Chen, 2009; Dupo and Barrion, 2009). *Nilaparvata lugens* is a ‘sucking pest’ which removes sap from the xylem and phloem tissues of the rice stem (Liu et al., 2010a). Severely damaged rice plants desiccate through the effects of feeding and ovipositor damage, a condition known as ‘hopper burn’ (Du et al., 2009). *Nilparvata lugens* is also a vector of rice virus diseases, such as ‘grassy stunt’ (Khush and Ling, 1974; Dyck and Thomas, 1979; Sōgawa, 1982; Li et al., 2011). *Nilaparvata lugens* populations fluctuate in response to changing environmental conditions, both physical (abiotic) and biotic, and can lead to pest outbreaks (Win et al., 2011). In general, *N. lugens* is endemic to the Asian sub-tropical region, though its range can expand temporarily every summer as far north as Japan and South Korea through long-distance migrations from the tropics (Sōgawa, 1982; Gurr et al., 2011). As tropical species experience less seasonal variation in temperature they generally have narrower
thermal tolerances compared with temperate species (Ghalambor et al., 2006; Deutsch et al., 2008; Bonebrake and Deutsch, 2012).

Much of the previous research on *N. lugens* has focused on the effect of rearing at different constant or variable temperatures on development and fecundity (Mochida and Okada, 1979; Krishnaiah et al., 2005) and on the impact of variation in the dietary composition of resistant cultivars on reproductive output (Sōgawa, 1982; Cheng, 1985; Cohen et al., 1997). By comparison, the effects of sub-lethal heat stress on development and reproduction have received little attention but are likely to become more important in a scenario of climate warming. The mean summer day time high temperature in China varies from 37 to 41°C (Chen and Zhao, 1999) and can rise to 50°C in some sub-tropical countries (Giese, 2011). Temperatures in this range are of interest because a recent study on *N. lugens* (Piyaphongkul et al., 2012a) found that nymphs were less heat tolerant than adults and concluded that in some parts of its distribution and under current climatic regimes, juvenile stages of *N. lugens* could become immobilised through heat stress and might be killed by high temperature exposure. Even though insects may survive thermal stress, there may however, be sub-lethal effects on key processes that would impact negatively on population abundance, and hence the pest status of species such as the brown plant hopper. This raises the interesting question of whether insects living in tropical areas are sufficiently heat tolerant to survive under current conditions and if they can also adapt to the more stressful climatic regimes that may be experienced in the future.

Using knowledge gained on the upper lethal temperatures of nymphal and adult *N. lugens* (see chapter 4), this study investigated the effects of sub-lethal high temperatures applied at different life cycle stages on the subsequent development, reproduction and longevity.
6.3 Material and methods

6.3.1 Insect materials

Adults of *N. lugens* were originally collected from the MARDI Research Station at Pulau Pinang in Malaysia. All insects in the stock culture and before and after experiments were reared on *Oryza sativa* L. cv. TN 1 at the maximum tillering stage, in cages or perspex boxes covered with 1.22 mm ventilation mesh at 16:8 L:D and 23 ± 0.5°C. Newly-hatched first-instar nymphs (within 48 h of hatching) and unmated adults (30-35 days old) were used in the experiments. All high temperature exposures were carried out in a programmable alcohol bath (Haake Phoenix 11 P2; Thermo Electron Corp., Germany) to an accuracy of ± 0.5°C.

To investigate the effects of sub-lethal high temperature on development and fecundity of *N. lugens*, insects were exposed at their upper lethal temperature (ULT$_{50}$). The ULT is determined by exposing insects at progressively higher temperatures and recording the mortality at each temperature. The ULT$_{50}$ is the estimated temperature at which 50% of the population is killed (Hazell et al., 2010a).

6.3.2 Effect of sub-lethal high temperatures on development and longevity

A sample of 150 newly-hatched first instar nymphs were warmed from 20°C at 0.5°C min$^{-1}$ to their ULT$_{50}$ (41.8°C), held for 2 min and then cooled at the same rate back to 20°C; preliminary experiments had indicated the time required for nymphs to be held at the ULT$_{50}$ to experience the desired exposure temperature. When insects are heated or cooled, for example, in an alcohol bath, there is a time delay between the bath reaching the set temperature and the insects achieving
thermal equilibrium at this temperature. This lag time is dependent on the thermal properties of the exposure system (McNabb and Wake, 1991) and in general, larger insects will take longer to reach thermal equilibrium with the surrounding environment (Digby, 1955; Forsman, 2000; Davidowitz et al., 2003; Tanaka, 2005).

From the surviving population a sample of 50 nymphs was placed individually on rice seedlings in Perspex boxes in the standard rearing conditions. A control group of 50 first instar nymphs were held individually in the same conditions. Daily observations were made to record the time taken to moult to adult and total longevity in the treatment and control groups. As the gender of the treated and untreated insects could not be determined at the first instar stage, the male and female sample sizes were not equal. A split-plot method was used to determine the main effects of treatment on the development and longevity of *N. lugens* using temperature treatment and sex as fixed factors in SPSS 17.0 software. In the split plot design, sex was a split plot factor within the temperature treatment.

6.3.3 Effects of sub-lethal high temperatures on fecundity

6.3.3.1 Nymphs

A sample 200 of newly-hatched first instar nymphs were heated from 20°C at 0.5°C min⁻¹ to their ULT₅₀ (41.8°C), held for 2 min, and then cooled back to 20°C at the same rate. Each surviving nymph was maintained individually in a Perspex rearing box containing a rice seedling. After moult ing to adult, 20 treated females and males were randomly selected and transferred as pairs into separate rearing boxes with a rice seedling and maintained in the standard rearing conditions.
Fecundity was measured by counting the number of emerging first instar nymphs at daily intervals until there was no further emergence.

6.3.3.2 Adults

A sample of 600 newly-hatched first-instar nymphs were reared together in a number of Perspex boxes containing rice seedlings until the late fifth instar, after which males and females were reared separately on rice seedlings to obtain unmated adults. For each mating combination, 100 adult virgin males and females were heated from 20°C at 0.5°C min⁻¹ to their ULT₅₀ (42.5°C), held for 6 min and then cooled back to 20°C at the same rate. From the surviving populations and a control population of the same age, 20 randomly selected pairs were established for each of three mating combinations: treated male x treated female, treated male x untreated female, and untreated male x treated female.

The control group was created by allowing nymphs to develop from first to fifth instar after which the sexes were separated; 20 male and female pairs were taken from this stock and then allowed to mate and oviposit under the same conditions. Fecundity was measured in the same way as in the experiment with first instar nymphs.

All data were analysed by one-way analyses of variance (ANOVA) to test for the effect of treatment on the number of emerged nymphs between treated nymphs and treated adults, and among adult mating combinations. Where significant differences occurred, the data were further analysed using Tukey's honest significance difference post-hoc test.
6.4 Results

6.4.1 Effect of sub-lethal high temperatures on development and longevity

When first instar nymphs were exposed at their ULT\textsubscript{50} of 41.8°C mean times required to complete nymphal development increased from 15.2 ± 0.3 (n = 31) and 18.2 ± 0.2 (n = 19) days for male and female nymphs to 18.7 ± 0.2 (n = 21) and 19.0 ± 0.2 (n = 29) days respectively in the treated insects. Exposure at the first instar increased the longevity of adult females (from 20.6 ± 1.1 to 24.4 ± 1.0 days), but adult males were unaffected (longevity of 25.7 ± 1.0 and 23.5 ± 1.1 days for control and treated insects); however, mean development time of treated males was shorter than that for the control males. Mean total longevity was also increased in female insects (from 38.8 ± 1.0 to 43.4 ± 1.0 days), but the lifespan of male insects was similar between the control and treated males (40.9 ± 0.9 and 42.2 ± 1.1 days).

The increase in mean development time from nymph to adult after exposure at the ULT\textsubscript{50} was significant (F\textsubscript{1, 96} = 64.64, p < 0.001), with a difference between the sexes (F\textsubscript{1, 96} = 35.68, p < 0.001) and in the interaction between the temperature treatment and sex (F\textsubscript{1, 96} = 25.40, p < 0.001). By comparison, there was no difference in adult longevity between the control and treated groups (F\textsubscript{1, 96} = 0.53, p = 0.470), nor between the sexes (F\textsubscript{1, 96} = 3.62, p = 0.060), but the interaction between the temperature treatment and sex was significant (F\textsubscript{1, 96} = 7.34, p = 0.008). There was a significant effect of temperature on total longevity (F\textsubscript{1, 96} = 8.76, p = 0.004), but no difference between the sexes (F\textsubscript{1, 96} = 0.24, p = 0.628), nor in the interaction between the treatment and sex (F\textsubscript{1, 96} = 2.65, p = 0.107).
The range of times required for nymphs to complete development to adult is shown in Figure 6.1A and 6.1B. Whilst the overall range of treated males (17-20 days) and treated females (16-21 days) was similar to that of the control groups (13-19 days for males and 17-20 days for females), within these ranges, the treated insects generally took longer to complete nymphal development in both males ($F_{1,50} = 66.25, p < 0.001$) and females ($F_{1,46} = 6.96, p = 0.011$).

![Figure 6.1](image)

**Figure 6.1** Range of development times for the nymphal stages of *N. lugens* after exposure at the ULT$_{50}$. $N = 50$ for control (31 male and 19 female) and treatment (21 male and 29 female) groups.
The impact of exposure of first instar nymphs at the ULT_{50} temperature on development persisted into the adult stage; whilst the range of adult lifespans were again similar for treated females (8-31 days) and controls (13-30 days), the treated insects lived longer (F_{1, 46} = 5.95, p = 0.019, Figure 6.2B). Treated males, however, did not live as long as the control group (10-30 days and 14-35 days respectively, F_{1, 50} = 1.97, p = 0.167, Figure 6.2A).

**Figure 6.2** Range of development times for adults of *N. lugens* after exposure as first instar nymphs at the ULT_{50}. N = 50 for control and treated groups (gender ratio as in Figure 6.1).
6.4.2 Effect of sub-lethal high temperatures on fecundity

6.4.2.1 Treated nymphs vs treated adults

After exposure at the ULT$_{50}$ of 41.8 and 42.5°C at the first instar and adult stage respectively, mean egg production per female decreased from 403.8 ± 13.7 in the untreated control to 128.0 ± 16.6 (treated nymph male x treated nymph female) and 169.3 ± 14.7 (treated male x treated female) ($F_{2, 57} = 62.12$, $p < 0.001$, Figure 6.3), with a range of 267–627 eggs per female in the control, 34–317 in the treated nymph group and 84–326 in the treated adult group. Overall, mean egg production was most reduced when insects were exposed as first instar nymphs (31.7% of control group), than when both sexes were exposed as adults (reduction to 41.9% of control). There was however, no difference in mean egg production between treated nymph male x treated nymph female and treated male x treated female ($p = 0.278$).
Figure 6.3 Mean number of eggs per female after exposure of first instar nymphs and adults of *N. lugens* at their ULT$_{50}$. N = 20 pairs for each mating combination. Mean values with the same letter are not significantly different at p < 0.05 level.

6.4.2.2 Treated adult mating combinations

For the three mating combinations after exposure of adults at the ULT$_{50}$ of 42.5°C the mean number of eggs produced per female were: 169.3 ± 14.7 (treated male x treated female, range 84-326), 249.6 ± 21.3 (treated male x untreated female, range 75-436) and 233.4 ± 17.2 (untreated male x treated female, range 94-412); $F_3, 76 = 25.47$, with all adult mating combinations producing significantly fewer viable eggs than the control, (p < 0.001, Figure 6.4). Overall mean
egg production was most reduced when both sexes had been exposed as adults (reduction to 41.9% of control), with less effect when only one sex was exposed as an adult (61.8% for treated male and 57.8% for treated female compared with the control group).

**Figure 6.4** Mean number of eggs per female after exposure of adults of *N. lugens* at their ULT$_{50}$. N = 20 pairs for each mating combination. Mean values with the same letter are not significantly different at p < 0.05 level.

*Nilaparvata lugens* produced viable eggs in all mating groups that included insects exposed at their respective ULT$_{50}$ temperatures (Figure 6.5). For all the treatment groups there was however, some delay until the first egg hatched and the range of egg development times was also extended
in all the treated groups: 11-16 days for treated nymphs, 10-21 days for treated adult male and female, 11-16 days for treated male x untreated female, 10-16 days for untreated male x treated female, compared with 9-14 days in the control; all treated groups were significantly different to the control (F_{4, 95} = 10.62, p < 0.001), but there was no difference between any of the treated groups.

**Figure 6.5** Range of egg development times after exposure of first instar nymphs and adults of *N. lugens* at their ULT_50_. N = 20 pairs for each mating combination.
6.5 Discussion and conclusions

Climate change operates on a global scale with wide-ranging and interrelated impacts across the social-economic-environmental interface (Leary and Kulkarni, 2007). A greater understanding of the effects of climate warming on agricultural and natural ecosystems will inform policies aimed at mitigating risks, particularly with regard to ectothermic organisms for which temperature is an important determinant of development, survival and distribution (Casey, 1992; Fox and Morin, 2001; Frazier et al., 2006; Sanuy et al., 2008; Angilletta Jr. et al., 2010). Insects have evolved a range of behavioural, physiological and biochemical adaptations to survive both seasonal and more acute fluctuations in temperature (Overgaard et al., 2008), but there are limits above and below which species cannot survive. A recent study with the brown planthopper *Nilaparvata lugens* found that around 50% of first instar nymphs were killed by a brief exposure at 41.8°C (ULT\textsubscript{50}) and a similar proportion of adults at 42.5°C; both life cycle stages were immobilized by heat stress at lower temperatures (Piyaphongkul et al., 2012a; see chapter 4). Whilst lethal temperatures provide estimates of the limits to survival, it cannot be assumed that individuals that survive at temperatures close to these limits are unaffected by the exposure (Bale, 1996). This study focused on the effects of sub-lethal high temperature exposure on the development and reproduction of *N. lugens*, a major pest of rice in tropical Asia.

After exposure of first instar nymphs at the ULT\textsubscript{50} of 41.8°C development time to adult was significantly increased in both male and female *N. lugens*. The combination of nymphal development time and adult longevity resulted in an overall extension of the total life span of females but not males. A number of studies that have shown that males and females of several insect species differ in absolute performance capacities (e.g. consumption of resources,
locomotor ability, duration of stress tolerance) when living under favourable (i.e. non-stressful) conditions (Milkman, 1963; Lailvaux et al., 2003; 2004; Lailvaux and Irschick, 2007). As temperature is known to have a major influence on various ‘rate-based’ processes in ectotherms (Lailvaux and Irschick, 2007), the data suggest that there may be inherent differences in the thermal biology of males and females, or that they are differentially affected by exposure to high temperature. The results from this study also support the view that sub-lethal high temperatures can have a negative impact on insect development, especially at temperatures close to the upper thermal limit (Howe, 1967; Bale and Hayward, 2010; Muller and Obermaier, 2012). The physiological explanation for impeded development following high temperature stress may be related to deleterious effects on respiratory metabolism (Davidson, 1944; Frazier et al., 2001; Nespolo et al., 2003; Woods and Hill, 2004; Harrison et al., 2010; Contreras and Bradley, 2011) or interference with the synthesis of hormones involved in the moulting process (Okasha, 1968; Lekovic et al., 2001).

As the eggs of *N. lugens* are laid in plant tissue, it is not possible to determine accurately the number of viable eggs laid, as some eggs would be destroyed when dissected out of the rice stems. Emergence of first instar nymphs was therefore used as an indicator of reproductive output. High temperature stress exerted a number of sub-lethal effects on reproduction in *N. lugens*: fewer nymphs emerged from eggs, the period of egg development was extended, and some nymphs were unable to moult to the second instar. An important factor that may contribute to the negative effects of high temperature stress on both development and reproduction in *N. lugens* concerns the role of the intracellular yeast-like symbiotes (YLS). In *N. lugens* and *Laodelphax striatellus* the YLS are contained in the fat body and transmitted transovarially
between generations (Noda et al., 1995). The YLS are reported to play an important role in the abdominal segmentation and differentiation of planthopper embryos (Lee and Hou, 1987) and synthesise essential amino acids (that are vital for normal development) to compensate for variable amino acid availability in different plant hosts (Chen et al., 2011). Exposure of newly hatched nymphs of *L. striatellus* for 3 days at 35°C reduced the number of YLS by approximately 90% (Zhang et al., 2008). The same treatment applied to nymphs of *N. lugens* for 3 days destroyed the YLS which in turn impeded development and ecdysis (Chen et al., 1981). Similarly, exposure at 32°C of 3 day-old adult females of *N. lugens* containing fully developed ovaries reduced the number of YLS and lowered fecundity (Hou and Lee, 1984; Lee and Hou, 1987).

In a study on the pine false webworm *Acantholyda erythrocephala*, eggs failed to hatch at around 30°C (Lyons, 1988). It is possible that the secretion of hormones from neurosecretory cells associated with egg production is inhibited by a direct heat exposure (Okasha, 1970), but after transfer to favourable conditions, the reproductive activities are resumed in both males and females, but with a net reduction in overall fecundity. High temperature exposure may also reduce mating success, sperm viability and oviposition, all of which would impact negatively on generation-to-generation population abundance (Reynoldson et al., 1965; Harcourt, 1969). Also, whilst the effects of sub-lethal heat stress on *N. lugens* reported here arose from very brief exposures, in nature, the time periods involved would be much longer, unless the insects showed some form of avoidance behaviour. For example, large leaves of the host plants of *Manduca sexta* L. became hotter during the day than smaller leaves such that by selecting smaller leaves for oviposition, the thermal buffering of extreme temperatures would increase egg survival and
successful hatching (Potter et al., 2009). A further consideration is that populations reared under laboratory conditions over long periods of time and multiple generations (with periodic refreshment with wild stock) may become increasingly different from natural populations through genetic bottlenecks (Gullan and Cranston, 2010). However, as population of *N. lugens* had been in culture for less than two years (and completed 11-12 generations), such effects are unlikely with the studied colony. It is also recognised that the effects of extreme exposures associated with climate change will most likely be revealed over longer term timescales and be subject to important interactions with other physical and biological factors (Parmesan et al., 2000; Thibault and Brown, 2008).

With these provisos in mind, the results from this study can be placed in a wider ecological context. Based on climatic data from various countries across the distribution of *N. lugens*, Piyaphongkul et al. (2012a) concluded that although mean temperatures were generally below the estimated ULT<sub>50</sub> values of nymphs (41.8°C) and adults (42.5°C) there were occasional extreme events that would overlap with these lethal temperatures, and that through heat-induced immobility at lower temperatures (at the CT<sub>max</sub>), insects may not be able to move away from potentially lethal exposure, or as has been identified in this study, deleterious effects of reproduction may occur. When insects are heated (or cooled) at rates that are faster than those experienced in nature, the observed mortality (or other deleterious effects) may be caused by the range of temperatures experienced, the rate of change, the most extreme temperature experienced or a combination of all factors. When adult *N. lugens* were heated at 0.5°C min<sup>-1</sup> to determine the ULT<sub>50</sub> (42.5°C), no insects were killed until exposure at 42°C (Piyaphongkul et al., 2012a; see chapter 4). As the same rate of warming was used in these experiments it seems reasonable to
conclude that neither the change in temperature (approximately 20°) nor the rate of increase in temperature are detrimental to survival per se – rather, it is the highest temperature experienced that impedes development and lowers fecundity.

Across the distribution of *N. lugens* in tropical Asia there is considerable variation in winter minimum temperatures and also the number of heat waves and more prolonged ‘hot spells’ in summer (UNFCCC, 2007). Extreme temperatures of over 45°C occur over the north-west part of the region during May-June, and several countries in this area have reported increasing surface temperature trends in recent decades. For example, the annual mean surface air temperature in Vietnam, Sri Lanka and India has increased by 0.30-0.57°C per 100 years (Lal et al., 2001). Moreover, regional climate change simulations for the 21st century by Atmosphere-Ocean General Circulation Models (AOGCMs) relative to the baseline period of 1961-1990 suggest that the area-average annual mean surface air temperature over land areas of Asia will be higher by 1.6 ± 0.2°C in the 2020s, 3.1 ± 0.3°C in the 2050s and 4.6 ± 0.4°C in the 2080s as a result of increases in the atmospheric concentration of greenhouse gas emissions (Giorgi and Francisco, 2000; Lal et al., 2001).

Importantly, the influence of temperature on insect development is related not only to the daily or monthly mean values, but also to the rate of temperature change that will sometimes include extreme exposures (Thibault and Brown, 2008; Müller and Obermaier, 2012). Whilst the experiments reported here and the previous study on the lethal and behavioural thermal thresholds (Piyaphongkul et al., 2012a) suggest that *N. lugens* may be adversely affected across parts of its current distribution by high temperature stress and progressive climate warming, for some insects a warmer climate may be beneficial, as has been observed with the range expansion
of the coffee berry borer (Hypothenemus hampei) (Jaramillo et al., 2011). As such, the opportunity to benefit from a warmer climate (or not to suffer deleterious effects) lies in part in the difference in temperature between the upper lethal limit (and the range over which sub-lethal effects occur) and prevailing and future climatic regimes, and the ability to exploit new areas where necessary resources are available, but temperature has previously been a barrier to establishment and residency. Indeed, whilst Piyaphongkul et al. (2012a) highlighted areas where N. lugens might experience thermal stress under current climates, and would be more likely to do so in warmer climate (unless acclimation occurred), there were also parts of the distribution where winter low temperatures currently prevent year-round survival, but which might become more favourable through climate change.

In summary, the results reported here indicate that the temperatures that kill around 50% of nymphs and adults of N. lugens also exert negative effects on development and longevity. The same exposures also lower fecundity through a combination of effects that operate through both of the sexes, in which the greatest effects occur when both males and females have experienced sub-lethal heat stress.

The data presented in this chapter have been published on:

Influence of Short Exposure to High Temperatures on Feeding Activity of the Brown Planthopper *Nilaparvata lugens*

7.1 Abstract

The effects of temperature stress on feeding behaviour were investigated in nymphs and adults of the brown planthopper *Nilaparvata lugens* on rice plants using the honeydew clock method. First instar nymphs and newly moulted adults were exposed at their respective LT$_{50}$ temperatures of 41.8 and 42.5°C and honeydew production observed in surviving insects over the following 15 (nymphs) and 21 (adults) days. Analysis of honeydew excreted by *N. lugens* over a period of 12 hours per day indicated a logistic regression relationship between honeydew excretion (mm$^2$) and time (days). The level of feeding activity as indicated by honeydew excretion increased in the order of treated male nymphs (♂tn) < treated female nymphs (♀tn) < control male nymphs (♂cn) < control female nymphs (♀cn) < treated adult males (♂ta) < treated adult females (♀ta) < control adult males (♂ca) < control adult females (♀ca). Short exposure to sub-lethal high temperature impacted negatively on the feeding activities of *N. lugens*, with significantly less honeydew produced by treated insects. There were however, no differences between treated males and females in either the nymphal or adult stages. Honeydew production measured during the late fifth nymphal instar for ♂cn, ♂tn, ♀cn and ♀tn were 2.9046, 1.0171, 4.0043 and 1.3646 mm$^2$/12 hr/day respectively, whilst the excretory rates for ♂ca, ♂ta, ♀ca and ♀ta were 43.6215, 14.9183, 93.7713 and 18.6637 mm$^2$/12 hr/day. These results are discussed in relation to the
effects of climate warming and extreme events on the population biology and distribution of \( N. \) 

\textit{lugens}.

\subsection*{7.2 Introduction}

An assessment of pest distribution and damage caused by insects and disease is needed to implement near real-time and future strategies of pest management and crop protection (Pedigo, 1995). The possible impacts of higher temperatures through climate warming on the feeding behaviour of crop pests is also an important issue in predicting future patterns of crop loss and yield. Despite a long history of research on insect pests across many species groups, information on the factors that limit feeding is sparse (Zvereva et al., 2010). Sap-sucking insects that feed on plant phloem are common worldwide, and can have greater negative effects on plant growth than leaf-chewing species (Buckley, 1987; James and Kelly, 2011). The relationship between sap-feeding insects and their host plants drives important ecosystem processes in various habitats (Dungan et al., 2007). The sugar-rich honeydew excreted by the insects is a vital energy source for other insects and fungi (Sasaki et al., 1996; Blüthgen et al., 2004; Ivon Paris and Espadaler, 2009). Typically, honeydew has lower levels of glucose and fructose and higher levels of complex sugars due to the enzymatic actions in the digestive system of sap-feeding species (Hendrix et al., 1992). Honeydew does not normally crystallize due to the reduced level of glucose. The mineral content of honeydew can be measured and this method is used to differentiate between honeydew produced by different species (Molyneux et al., 1990).
There have been a number of studies on honeydew production in different species including the pea aphid *Acyrthosiphon pisum* (Randolph et al., 1975), tea aphid *Toxoptera aurantii* (Baoyu and Chongsong, 2007), soybean aphid *Aphis glycines* (Wyckhuys et al., 2008), scale insect *Ultracoelostoma* sp. (Beggs et al., 2005; Dungan et al., 2007; James and Kelly, 2011), *Coelostomidia wairoensis* (Gardner-Gee and Beggs, 2007), *Coccus hesperidum* L. (Bogo and Mantle, 2000) and *Nilparvata lugens* (Begum and Wilkins, 1998; Chen, 2009; Ghaffar et al., 2011; Qiu et al., 2011). Most studies on honeydew produced by sap-feeing insects has however, focused on its nutritional value for their parasitoids and other natural enemies (Fuchsberg et al., 2007; Faria et al., 2008; Wäckers et al., 2008; Wyckhuys et al., 2008).

The brown planthopper *Nilaparvata lugens* is widely regarded as the most serious rice pest in Asia, affecting a wide range of economically grown rice crops that arose from the green revolution (Sōgawa, 1982; Saxena and Barrion, 1983; Visarto et al., 2006; Chen, 2009; Dupo and Barrion, 2009). Brown planthoppers cause damage to rice directly by inserting their stylet mouthparts into the phloem of the rice stem and imbibing the nutritive sap which is rich in sugars and amino acids (Yao et al., 2012; Zhang et al., 2004; Liu et al., 2010b). The phloem sap and nutrients are transported into the gut of *N. lugens* and absorbed into the body via midgut cells, whilst the excess sap after digestion is excreted as honeydew (Sōgawa, 1982; Begum and Wilkins, 1998; Kikuta et al., 2010). Feeding by a large number of *N. lugens* may result in drying of the leaves, wilting of the tillers, or death of the plant. The combined effects of feeding and ovipositor insertions lead to discolouration of plants, a condition known as "hopper burn" (Zhang et al., 2004; Du et al., 2009). *Nilparvata lugens* are also vectors of serious rice virus diseases such as grassy stunt and rice ragged stunt (Khush and Ling, 1974; Dyck and Thomas, 1979; Sōgawa,
1982; Gurr et al., 2011; Li et al., 2011). In general, *N. lugens* is endemic to the Asian tropical sub-region e.g. Thailand, Vietnam, Laos; however, they migrate northwards from the Indo-China peninsular every year to southern China in early summer (Otuka, 2008; 2009) and from southern China to central China, Japan and South Korea (Sōgawa, 1982; Catindig et al., 2009; Cheng, 2009; Watanabe et al., 2009; Gurr et al., 2011).

Outbreaks of *N. lugens* have threatened rice production and food security in Asia (Dyck and Thomas, 1979; Heinrichs and Barrion, 2004; Cheng, 2009; Watanabe et al., 2009; Chen et al., 2011; Bottrell and Schoenly, 2012). Many studies have examined the physiological and behavioural responses of *N. lugens* in relation to their insecticide resistance (Chelliah and Heinrichs, 1984; Bao et al., 2012), and also the use of rice varieties that are resistant to the pest (Khush, 1979; Alam and Cohen, 1998; Alagar et al., 2007; Chen et al., 2011). It has however, now been demonstrated that *N. lugens* is highly adaptable and under selection pressure can improve their performance on resistant rice varieties e.g. increases in survival, body weight, honeydew production and reproduction (Chen, 2009; Horgan, 2009; Gurr et al., 2011). Honeydew excretion can be used as a measurement of feeding activity and consequently, as an index for assessing the susceptibility or resistance to *N. lugens* of new rice varieties (Begum and Wilkins, 1998; Chen, 2009; Ghaffar et al., 2011; Qiu et al., 2011). Ghaffar et al. (2011) reported that *N. lugens* produced lower numbers of honeydew droplets when feeding on resistant varieties of rice, which correlates with previously published data about the feeding behaviour on resistant and susceptible cultivars. There is some evidence that *N. lugens* feed less and excrete less honeydew when feeding on rice plants deficient in nitrogen (Sōgawa, 1982). Chen (2009) also suggested that lower levels of free amino acid, lower concentrations of reducing sugar and higher
concentrations of flavonoid glycosides in resistant rice varieties could be related to the observed reduced feeding performance in *N. lugens*. Furthermore, Seo et al. (2010) showed by electrical penetration graphs (EPG) that *N. lugens* have difficulty accessing the phloem of resistant rice varieties. Little is known however, about the effect of climate change on the feeding behaviour of *N. lugens* particularly, the effects of exposure to extreme heat stress events that are associated with climate warming.

Temperature plays an important role in insect population dynamics (Muhamad and Fee, 1993; Way and Heong, 1994; Chapin III et al., 2000; Chown et al., 2010; Win et al., 2011), though insects possess a range of systems that restrict damage or depress metabolism under extreme temperatures (Huey and Kingsolver, 1993; Feder and Hofmann, 1999; Sørensen et al., 2003; Martin and Huey, 2008; Chown et al., 2010; Rezende et al., 2011). The surface air temperature warming effect across seasons is predicted to be lower in the tropics than at high latitudes (Heong et al., 1995; Lawrimore et al., 2001; Jansen et al., 2007) because the tropics are characterized by high year-round temperatures (Rosenzweig and Liverman, 1992). It has also been hypothesized from recent work that tropical species are already living close to their upper thermal limits (Deutsch et al., 2008; Tewksbury et al., 2008; Rezende et al., 2011; Piyaphongkul et al., 2012a), such that even a small change in temperature may have a negative impact on survival.

Meteorological records suggest that global warming affects both mean climatic parameters and the frequency of extreme meteorological events (Bell et al., 2000; Rosenzweig et al., 2001; Yan et al., 2002). For example, surface air temperatures in tropical sub-regions can increase to around 50°C in summer (Giese, 2011) and heat waves have become more frequent over the last century (Gornall et al., 2010). Population abundance of *N. lugens* fluctuates in response to the dynamic
abiotic and biotic conditions of their environment, leading to pest outbreaks (Win et al., 2011). However, it still remains unclear as to how abiotic stresses will affect *N. lugens* and its natural enemies, though some insight has been gained from recent studies (Bottrell and Schoenly, 2012). Bae et al. (1987) reported that the optimal temperature for egg hatch was 25°C and decreased below or above this temperature, and that development was impeded at 30°C or higher. In addition, higher temperatures affected *N. lugens* populations differently in different developmental stages (Heong et al., 1995), possibly due to the differential mortality of intracellular symbiotes (Bottrell and Schoenly, 2012). Exposure of nymphs and adults of *N. lugens* for brief periods of time at their ULT$_{50}$ (temperature that kills 50% of population) exerted negative effects on both development and reproduction (Piyaphongkul et al., 2012b). There are a number of possible explanations for such deleterious effects of heat stress on parameters of fitness including direct damage to somatic tissues or a reduced ability to feed or assimilate essential resources from the phloem diet. Against this background the main aim of this study was to examine the effects of sub-lethal high temperature exposure on the feeding behaviour of *N. lugens* by measuring honeydew production in different life cycle stages.

7.3 Material and methods

7.3.1 Plant

All experiments were carried out with *Oryza sativa* L. cv. TN 1 at maximum tillering stage because at this stage the increase of the tertiary tillers continues up to a certain point designated (De Datta, 1981). *O. sativa* cv. TN1 is also highly susceptible to all *N. lugens* biotypes and
carries no resistance genes against brown planthopper (Qiu et al., 2011). Plants were grown in a mix of three parts of ‘Levington’ M3 high nutrient compost (pH: 5.3-5.7, N280:P160:K350, Everris Limited, Epsilon House, West Road, Ipswich Suffolk, UK) and one part of ‘Silvaperl’ perlite (particle size 1.0-5.0 mm, William Sinclair Holdings PLC, Firth Road, Lincoln, UK). The rice seedlings were individually grown in plant pots (diameter, 3.5 and 5.5 cm, bottom and top, respectively; 5 cm in height, one seedling per pot) in a plant growth room under conditions of 16:8 L:D cycle, 30°C, and 70% RH.

7.3.2 Insects

Stock populations of N. lugens were provided by the MARDI Research Station at Pulau Pinang, Malaysia in 2010. Rearing conditions have been described previously by Piyaphongkul et al. (2012a; see chapter 4). The N. lugens colony had been in culture on Oryza sativa L. cv. TN 1 at maximum tillering stage for 22 months prior to the experiment. As each generation takes approximately 7 weeks at 23ºC, there would have been approximately 14 generations before the experiments were conducted. All rearing containers were kept in a quarantine room at 23 ± 0.5ºC, 16:8 L:D cycle. Experiments were carried out with 2 day old first instar nymphs and 18-20 day old unmated adult females and males.

7.3.3 Honeydew collection and analysis

Honeydew was collected using the method described by Ghaffar et al. (2011) and Kemp (2011) by using a filter paper technique and modified honeydew clocks. The base of a 14 cm diameter Petri dish was placed on the hour-hand spigot of a clock that rotated through 360º over a 12 hour period. A 125 mm diameter Whatman filter paper disk was pre-treated with 0.1% water soluble
bromophenol blue (Merck KGaA, Germany) and 0.01 M HCl (Sigma-Aldrich Company Ltd., UK), and placed onto the bottom of the Petri dish. A rice plant was suspended horizontally over the Petri dish containing the filter paper. The plant and honeydew clock system were contained within a ‘Bug dorm’ insect rearing cage (Bug dorm model 42260; 22 cm length x 22 cm width x 60 cm height – MegaView Science Co. Ltd., Fuya Road, Taichung, Taiwan). All experiments were carried in a quarantine room at 23 ± 0.5°C and 16:8 L:D cycle.

![Diagram of equipment](image)

**Figure 7.1** Apparatus used for quantifying honeydew produced by *N. lugens* on rice plants over time.

To study the effects of heat stress on the feeding behaviour of *N. lugens* 200 newly emerged nymphs (24-48 h old) and unmated adult females and males (15-20 days old) were collected from rice host plants. The nymphs and adults were then warmed from 20°C at 0.5°C min⁻¹ to their respective ULT₅₀ (41.8 and 42.5°C) and held at these temperatures for 2 min (nymphs) and 6 min (adults) based on the times required for the sample to equilibriate with the required temperature.
Insects used in experiments (n = 10 for both age groups) were selected at random from populations that survived exposure at the ULT$_{50}$. Control groups were starved on moist filter paper for 4 h before the experiment. Honeydew was collected over a 12 h period (08.00 to 18.00) for 15 (nymphs) and 21 (adults) days. Each filter paper was photographed after daily removal from the Petri dishes. The honeydew droplets appeared as blue spots on the filter paper. The area of each droplet was measured using a digital scanner and “Image J” software according to the following formula $\sum r^2$, where $r$ = radius of each honeydew spot (mm).

Data presented below represent the mean honeydew production per 12 h (± SE) expressed as the areas produced over the daily 12 hour observational periods. A standard linear regression method was used to predict the amounts of honeydew excretion/12 h over the duration of the experiment. A split-plot method was used to determine the main effects of temperature treatment on honeydew production by *N. lugens* using temperature and sex as fixed factors in SPSS 20.0 software. In the split plot design, sex was a split plot factor within the temperature treatment.

**7.4 Results**

7.4.1 Effects of sub-lethal high temperature exposure on feeding activity in nymphs

When first instar nymphs were exposed at their ULT$_{50}$ of 41.8°C, mean honeydew production/12h was lower in the treated groups compared with the control (Figure 7.2). Control nymphs showed low feeding activity during the first four days after emergence, whilst in treated nymphs there was no measurable output of honeydew until day 7. Honeydew production increased in both
treated and control nymphs over the duration of the experiment. Linear regression analyses showed that there was a significant relationship between honeydew area, mm² (Y) and the day (X), where \( Y = 0.067X - 0.278 \) (\( R^2 = 0.764, F_{1,148} = 478.89, p < 0.001 \)) and \( Y = 0.089X - 0.364 \) (\( R^2 = 0.745, F_{1,148} = 431.68, p < 0.001 \)) for treated male and female nymphs, and \( Y = 0.212X - 0.556 \) (\( R^2 = 0.905, F_{1,148} = 1413.74, p < 0.001 \)) and \( Y = 0.285X - 0.814 \) (\( R^2 = 0.890, F_{1,148} = 1195.39, p < 0.001 \)) for control male and female nymphs respectively. Thus in all sample groups there was a strong relationship between honeydew excretion and time, with a significant increase in production over the duration of the experiment.

**Figure 7.2** Honeydew production/12 h by nymphs of *N. lugens* feeding on rice plants for 15 days: A) control nymph males, B) control nymph females, C) treated nymph males and D) treated nymph females. N = 10 for each group.
Mean honeydew production by male and female treated and control nymphs over the duration of the experiment is shown in Table 7.1. Honeydew excretion differed significantly between treated and control nymphs on every day of the experiment and between the sexes on most days (both $p < 0.001$, Table 7.1). There was no interaction between temperature and gender over days 6-12 but this was significant over the last three days of observations.

**Table 7.1** Mean honeydew production (mm$^2$/12 h) by temperature-treated and control nymphs of *N. lugens* (n =10).

<table>
<thead>
<tr>
<th>T (day)</th>
<th>Honeydew production (mm$^2$)</th>
<th>F value, p</th>
</tr>
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<td></td>
<td>♂cn</td>
<td>♂tn</td>
</tr>
<tr>
<td>D4</td>
<td>0.3874</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>p = 0.002</td>
<td>p = 0.767</td>
</tr>
<tr>
<td>D5</td>
<td>0.4936</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.011</td>
</tr>
<tr>
<td>D6</td>
<td>0.6450</td>
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</tr>
<tr>
<td></td>
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<tr>
<td>D7</td>
<td>0.8077</td>
<td>0.04479</td>
</tr>
<tr>
<td></td>
<td>p &lt; 0.001</td>
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</tr>
<tr>
<td>D8</td>
<td>1.0133</td>
<td>0.1081</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>D9</td>
<td>1.1192</td>
<td>0.2001</td>
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<td></td>
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<td>p = 0.012</td>
</tr>
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<th>♀cn</th>
<th>♀tn</th>
<th>Treatment</th>
<th>Sex</th>
<th>T*S</th>
</tr>
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<tbody>
<tr>
<td>D10</td>
<td>1.4783</td>
<td>0.2612</td>
<td>1.6994</td>
<td>0.4125</td>
<td>F = 218.25</td>
<td>F = 4.83</td>
<td>F = 0.17</td>
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<tr>
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<td></td>
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<td></td>
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<td>p = 0.683</td>
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<tr>
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<td>2.1023</td>
<td>0.4559</td>
<td>F = 284.98</td>
<td>F = 7.58</td>
<td>F = 3.54</td>
</tr>
<tr>
<td></td>
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<td></td>
<td>p &lt; 0.001</td>
<td>p = 0.009</td>
<td>p = 0.068</td>
</tr>
<tr>
<td>D12</td>
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<td>0.4680</td>
<td>2.4941</td>
<td>0.7284</td>
<td>F = 241.36</td>
<td>F = 15.31</td>
<td>F = 1.99</td>
</tr>
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<td></td>
<td></td>
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<td>p &lt; 0.001</td>
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<tr>
<td>D13</td>
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<td>0.6185</td>
<td>3.0923</td>
<td>0.7215</td>
<td>F = 310.99</td>
<td>F = 19.82</td>
<td>F = 12.47</td>
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<td></td>
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<tr>
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<td>p &lt; 0.001</td>
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<td>p = 0.001</td>
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</tbody>
</table>

7.4.2 Effects of sub-lethal high temperature exposure on feeding activity in adults

The impact of exposure of adults at the ULT_{50} temperature (42.5°C) on feeding behaviour was similar to that observed with nymphs (Figure 7.3). The mean daily production of honeydew was lower on day 1 than on all subsequent days across all sample groups. The treated insects (male and female) produced less honeydew than the equivalent control, with untreated females having
the highest output. There was a significant relationship between honeydew excretion (Y) and the day (X) for both treated adult males and females, $Y = 0.6X - 2.086 \left( R^2 = 0.727, F_{1,208} = 552.696, p < 0.001 \right)$ and $Y = 0.785X - 2.656 \left( R^2 = 0.716, F_{1,208} = 524.489, p < 0.001 \right)$, and for the control groups ($Y = 1.561X - 4.726 \left( R^2 = 0.703, F_{1,208} = 493.464, p < 0.001 \right)$ for males, and $Y = 3.155X - 9.850 \left( R^2 = 0.496, F_{1,208} = 204.621, p < 0.001 \right)$ for females). As with nymphs, honeydew production increased significantly over time in all sample groups.

**Figure 7.3** Honeydew production/12 h by adult *N. lugens* feeding on rice plants for 21 days: A) control adult males, B) control adult females, C) treated adult males nymphs and D) treated adult females. N = 10 for each group.
Mean honeydew production/12 h over the 21 day observation period was significantly reduced in treated insects (p < 0.001 on 20 days) and males produced significantly less honeydew on most days (Table 7.2). Significant interactions between temperature treatment and gender (p < 0.05) were found in >50% of the daily observations.

**Table 7.2** Mean honeydew production (mm²/12 h) by temperature-treated and control adults of *N. lugens* (n = 10).

<table>
<thead>
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<th>T (day)</th>
<th>Honeydew production (mm²)</th>
<th>F value, p</th>
</tr>
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<tbody>
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<td>♂ ta</td>
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<td>D1</td>
<td>1.1260</td>
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<tr>
<td></td>
<td>♂ca</td>
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<tr>
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<td>p&lt; 0.001</td>
<td>p = 0.013</td>
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<td>D17</td>
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<td>10.9560</td>
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7.5 Discussion and conclusions

The feeding process of *N. lugens* on rice host plants consists of a number of sequential steps including orientation to the plant, labial (mouthpart) exploration of the rice stem surface, stylet penetration into rice tissue accompanied by salivation, phloem sap acquisition from vascular bundles and honeydew excretion (Sōgawa, 1982; Jung and Im, 2005). Feeding and honeydew
excretion is substantially reduced on resistant cultivars (Choi and Park, 1999; Chen and Cheng, 1979; Ghaffar et al., 2011, Li et al., 2011). Whilst insects generally have access to adequate water and carbohydrate sources (Wang et al., 2009; Johnson et al., 2011), higher temperatures above the optimum, and especially experience of extreme events, may have negative impacts on development and reproduction, or become lethal (Wang et al., 2009; Johnson et al., 2011).

Recent studies on *N. lugens* found that exposure at ULT$_{50}$ temperatures exerted such effects on both nymphs and adults, leading to the hypothesis that sub-lethal heat stress may interfere with feeding behaviour or dietary resource acquisition in surviving individuals. This study provides evidence to support this view. Thus high temperature exposure reduced subsequent feeding activity and honeydew production in both life stages of *N. lugens* and these effects persisted over 15 and 21 days respectively; honeydew excretion also increased over these periods of observation and differed between sexes (Tables 7.1 and 7.2). Across all experimental groups honeydew production increased in the order treated male nymphs < treated female nymphs < control male nymphs < control female nymphs < treated adult males < treated adult females < control adult males < control adult females. The amount of honeydew excreted by females in the treated nymph and adult groups was 3-4x lower than in the equivalent control groups. Similarly, treated nymphal and adult males produced around 2-3x less honeydew than the respective controls.

A study by Choi and Park (1999) also observed that *N. lugens* nymphs produce less honeydew than adults and that daily output was higher in females (43.6 – 55.2 mm$^2$/female/day) than males (16.6-25.3 mm$^2$/female/day). Honeydew production by adult females feeding on the susceptible TN1 cultivar was higher in this study than in previous investigations, but this may be related, at least in part, to the method used to quantify honeydew output. For example, Cheng (1985)
measured honeydew production from newly moulted adult females on susceptible rice cultivar TN1 and reported a value of 27.4-37.2 mm²/female/day by using a filter paper technique. Similarly, Begum and Wilkins (1998) examined the feeding activity of female *N. lugens* at the same stage of development and on the same rice cultivar and recorded honeydew output of 26.5-32.4 mg/sachet/day using a parafilm sachet technique. In addition, Mollah et al., (2011) investigated the feeding response of four day old adult females on TN1 and found honeydew excretion of 49 mg/sachet/day using the same parafilm sachet technique. The differences in honeydew production in different studies (including this one) are most likely related to the different methods used to quantify honeydew excretion and the age of the insects when the observations were made.

There are a range of possible effects of extreme temperatures that may explain the reduction in feeding or production of honeydew observed with the heat stressed *N. lugens*. Firstly, high temperatures denature proteins (Salvucci et al., 2000) and any effect on the mid gut proteins that facilitate uptake and transportation of sugars would be deleterious (Price et al., 2007; Kikuta et al., 2010) as these sugar transport proteins also appear to play a crucial role in sugar metabolism and energy acquisition (Wood and Trayhurn, 2003; Kikuta et al., 2010). Also, many important protein enzymes found in insects function as catalysts in cells and regulate metabolism (Thompson and Lee, 1994). Heat stress may modify the structure of sugar transporting proteins and nucleic acids by disrupting weak interactions such as vander Waals, ionic and hydrogen bonds that stabilize conformation (Neven, 2000). Although it has been reported that some proteins can return to a functional conformation after denaturation, little is currently known regarding such mechanisms in insects (Kanamori et al., 2010; Kikuta, 2010). If there is a decrease
in energy release from the sugar-rich phloem diet because of heat stress, this may result in delayed development, lower fecundity and suppressed population build-up (Bae et al., 1987; Piyaphongkul et al., 2012b).

A second area of consideration is the close association in *N. lugens* with yeast-like symbiotes (YLS) located in fat body cells (Tang et al., 2010) that are vertically transmitted to the next generation via transovarian infection and proliferate by asexual budding (Noda et al., 1995; Lu et al., 2004). YLS have been postulated to play a number of important roles in *N. lugens* such as adaptation to resistant rice varieties (Lu et al., 2004; Chen, 2009), supply of nutrients required for normal embryonic and postembryonic development (Lu et al., 2004), and involvement in abdominal segmentation and differentiation of embryos for reproduction in adults (Hou and Lee, 1984). Sasaki et al. (1996) suggested that uric acid synthesized by *N. lugens* after ingesting excess amino acids is stored in tissues rather than excreted and then recycled with the aid of its endosymbionts. YLS are symbiotically associated with every developmental stage of *N. lugens* and play an important role in nitrogen metabolism (Hongoh and Ishikawa, 1997; Lu et al., 2004). The numbers of YLS in *N. lugens* are however, deleteriously affected and eliminated by high temperature stress (Chen et al., 1981; Noda et al., 1995). In nature, surface air temperatures may increase above 40°C for short periods in many areas across the distribution of *N. lugens* e.g. 37-41°C in China (Chen and Zhao, 1999), 47.8°C in India, 37.4-38.9°C in South Korea, 37.5-40°C in Bhutan, 42.5-47.2°C in Burma, 46.7°C in Nepal, 40.2-42.2°C in Philippines, and 40.4-42.7°C in Vietnam (MHERRERA, 2012). These high temperatures overlap the known lethal temperatures of *N. lugens* (Piyaphongkul et al., 2012a) and most likely their YLS, thus leading to reduced dietary metabolism and honeydew production, with related negative effects on fecundity, egg
viability and development (Piyaphongkul et al., 2012b; see chapter 6). Finally, there is the possibility that the YLS have a role with pathogen-related proteins to reduce defense reactions in plants in response to insect feeding (Zhang et al., 2004). Thus heat stress may destroy the YLS, allowing the plant to mount a defense response, and in turn, reduce the feeding activity of *N. lugens*.

In summary, this study has shown that exposure at high temperatures that induce around 50% mortality in nymphs and adults of *N. lugens* also reduce the feeding activity of survivors as measured by the production of honeydew. As insects that survive such exposures are known to produce fewer viable eggs and have a much reduced fecundity (Piyaphongkul et al., 2012b), it is likely that a combination of a decrease in feeding activity or interference with the dietary metabolic functions of proteins and endosymbionts contribute to the observed deleterious effects on development and reproduction. These observations are of ecological and agricultural importance as temperatures capable of inducing these negative effects occur across many areas of the current distribution of this important pest. As concluded in recent related studies on *N. lugens* (Piyaphongkul et al., 2012a; b), the impact of climate warming and extreme climatic events may be to make areas of the current distribution more or less favourable without necessarily changing the overall pest status of the brown planthopper world-wide.
CHAPTER 8

General Conclusions

Rice and its derived products is the main staple food for over half of the world’s population and is the most rapidly growing food resource in Asia and Africa in terms of the area under cultivation (Diouf, 2003). The intensification of rice production has required the extensive use of pesticides leading to water pollution and the destruction of the natural enemies of rice pests (Holt et al., 1992; Heong and Samson, 2012). The brown planthopper *Nilaparvata lugens* became the most serious insect ‘sucking pest’ of rice in Asia following the ‘green revolution’ (Catindig et al., 2009; Bottrell and Schoenly, 2012). Although there have been extensive studies on the biology, ecology and pest status of *N. lugens*, relatively little is known about its thermal tolerance or the potential impacts of global climate change on its development, reproduction, feeding activity and distribution. This study is the first to investigate the effects of thermal stress on *N. lugens*. Whilst one focus of this research was to determine the effects of such thermal stress on the pest status of *N. lugens*, a further objective was to investigate the extent to which tropical insects are able to acclimate to higher temperatures, as might be experienced in various scenarios of climate warming.

The accurate determination of temperature is vital in ecophysiological experiments, particularly those that focus on behavioural responses to temperature. The experiments described in chapter 2 illustrate that thermal gradients can occur even within small exposure environments, hence the need to conduct calibration measurements so that activity thresholds such as the critical thermal minimum and maximum and coma temperatures can be determined with accuracy.
A further source of error in thermal biology experiments concerns the time required for the exposure environment and sample organisms to equilibriate at the same temperature, the ‘lag time’ (chapter 3). The first main conclusion is that body size has a significant influence on the time required for an organism to achieve thermal equilibrium with its environment and that smaller species reach this state most quickly. Also, the type and number of containers within which insects are confined affects the time required to reach thermal equilibrium, but these factors appear to have been rarely considered in thermal biology experiments. When a sample of insects was exposed in a plastic tube within a glass tube or directly within a glass tube, different periods of time were required to reach thermal equilibrium, related to different rates of transfer of thermal energy (Mattos and Gasper, 2002).

The research described in this thesis on the effects of high temperature exposure and thermal stress on *N. lugens* can be summarized in four main conclusions. Firstly, although *N. lugens* can live at temperatures close to its upper thermal limits, occasional heat stress is detrimental to survival and likely to influence the current and future distribution (chapter 4). Secondly, *N. lugens* has less acclimatory ability to increase heat tolerance than cold tolerance (chapter 5). Thirdly, sub-lethal heat stress has a negative impact on development of eggs and later developmental stages and on fecundity (chapter 6). Lastly, exposure of *N. lugens* at their ULT$_{50}$ reduces the feeding activity of survivors, as measured by the production of honeydew (chapter 7). Whilst these conclusions are presented here as separate statements, they are clearly interrelated; for example, a reduction in feeding and dietary resource acquisition is likely to be a partial explanation for the observed effects on development and fecundity. The following sections explore these conclusions in more detail.
8.1 Upper thermal limits of *N. lugens* in natural environment

The experiments on the upper activity thresholds and thermal limits (chapter 4) obtained data on three indices: critical thermal maximum ($CT_{\text{max}}$), heat coma temperature (HCT) and upper lethal temperature (ULT). These results indicated that across all measurements, nymphs were less heat tolerant than adults. The accurate measurement of upper thermal limits (and whether individuals can recover from exposure at the HCT) is important information as it provides a basis for assessing the likelihood of species such as *N. lugens* experiencing thermal stress under current climate conditions and the risk posed by higher temperatures that may occur through progressive climate warming.

*Nilaparvata lugens* are generally found in tropical sub-regions where variation in surface air temperature between seasons is less than in other Asian sub regions and there is a characteristically high year-round surface air temperature (Mazur, 2011). Moreover, there has been an increasing frequency of extreme ‘high temperature’ events in summer in different parts of the distribution of *N. lugens* (Giese, 2011). Collectively, the upper thermal limit data indicate that *N. lugens* is already living close to its upper thermal threshold across part of its distribution. Future higher temperatures are likely to become an important limiting factor as the insects may be rendered immobile by heat stress or killed by extreme high temperature. This conclusion is further supported by the fact that the data obtained in these experiments relate to very brief exposures (typically less than hour), whereas in nature, once an individual is immobilised by heat stress, there is no other escape route. There are though other factors that may affect this conclusion, including the possibility of intraspecific variation in thermal tolerance in
geographically distinct populations and as yet, an unknown level of acclimatory ability (Fangue et al., 2006). This latter aspect was therefore investigated and is discussed in the next section.

8.2 *Nilaparvata lugens* has less ability to increase heat tolerance than cold tolerance

The studies in chapter 5 investigated the effects of acclimation at 15 and 30°C on the lower (CTₘᵟᵦₖ, CCT and LLT₅₀) and upper thermal limits (CTₘₐₓ, HCT and ULT₅₀) comparing with the standard rearing regime of 23°C. The thermal tolerance limits of both nymphs and adults changed significantly with acclimation and were correlated with rearing temperature.

In relation to the focus of this project on heat stress, an important observation to emerge was that although acclimation at 30°C increased the CTₘₐₓ, HCT and ULT₅₀, heat tolerance increased less than cold tolerance, when *N. lugens* were reared at 15°C. When these comparisons of acclimatory ability are interpreted in an ecological context, the data confirm that there is some ability to survive at the low temperatures experienced in temperate sub-regions e.g. China, Japan and South Korea; and although year round survival is not currently possible in these areas because of winter temperatures, a combination of acclimation and higher temperatures through climate warming may change this situation. Such changes in distribution linked to climate change will also have an impact on the agricultural importance of *N. lugens* across its current distribution, as a warmer climate may make some habitats more favourable, because of increased winter survival, and others less so, because of extreme summer heat stress.
The outcomes of experiments on upper lethal limits and irreversible heat coma are expressed in terms of the ratio of mortality to survivorship i.e. at the ULT<sub>50</sub>, approximately 50% of an exposed population will live or die. However, it cannot be assumed that the survivors are as ‘ecologically fit’ as individuals that have not experienced the same stress. For this reason, experiments were carried out on the effects of exposure at sub-lethal levels of heat stress.

8.3 Sub-lethal heat stress impedes development and lowers fecundity in <i>N. lugens</i>

Exposure of nymphs and adults of <i>N. lugens</i> at their respective ULT<sub>50</sub> temperatures exerted negative effects on development and longevity (chapter 6). Insects that had experienced this heat stress took longer to complete their nymphal development and this exposure also extended the total life span of females. A further negative consequence of impeded development is that insects may be killed by natural enemies before reaching sexual maturity and reproducing. The physiological explanation for this disruption to development may be related to deleterious effects on respiratory metabolism (Harrison et al., 2010; Contreras and Bradley, 2011) or interference with the synthesis of hormones involved in the moulting process (Lekovic et al., 2001).

The same sub-lethal exposures also lowered fecundity. This effect occurred when <i>N. lugens</i> was exposed as nymphs or adults, with the greatest effect observed when both males and females had been subject to sub-lethal heat stress. An important factor that may contribute to the negative effects of high temperature stress on both development and reproduction in <i>N. lugens</i> concerns the role of the intracellular yeast-like symbiotes (YLS) that are contained in the fat body (Noda et al., 1995; Tang et al., 2010). The YLS are reported to play an important role in the abdominal
segmentation and differentiation of brown planthopper embryos (Lee and Hou, 1987) and also contribute to synthesis of essential amino acids that are vital for normal development. A fully functioning complement of YLS may therefore compensate for the variable amino acid availability in different host plants (Chen et al., 2011). The data suggest that various activities such as egg development, oviposition, metamorphosis and mobility are all adversely affected at temperatures above 40°C and these effects may be related to the loss of large numbers of YLS (Chen et al., 1981; Hou and Lee, 1984; Lee and Hou, 1987; Zhang et al., 2008).

8.4 Exposure of N. lugens at their ULT$_{50}$ reduces feeding activity

Sub-lethal heat stress may also impact on feeding behaviour and any reduction in feeding is likely to lead to slower development and lower fecundity. In sap-feeding species such as N. lugens, the production of honedew as an excretory product is commonly used as a measure of phloem acquisition. The experiments described in chapter 7 indicate that following a brief exposure to heat stress, there is a marked reduction in phloem intake.

There are a number of possible explanations for the effects of extreme temperatures on the feeding behavior and honeydew production in heat-stressed N. lugens. Firstly, high temperatures denature proteins (Neven, 2000; Salvucci et al., 2000) and any effect on the mid gut proteins that facilitate uptake and transportation of sugars would be deleterious (Price et al., 2007; Kikuta et al., 2010), as these sugar transport proteins also appear to play a crucial role in sugar metabolism and energy acquisition (Wood and Trayhurn, 2003; Kikuta et al., 2010).
Secondly, heat stress may destroy the YLS which play a number of important roles in *N. lugens*, such as adaptation to resistant rice varieties (Lu et al., 2004; Chen, 2009), supply of nutrients required for normal embryonic and postembryonic development (Lu et al., 2004), and involvement in abdominal segmentation and differentiation of embryos for reproduction in adults (Hou and Lee, 1984). The numbers of YLS in *N. lugens* are however, deleteriously affected and eliminated by high temperature stress (Chen et al., 1981; Noda et al., 1995), thus leading to reduced dietary metabolism and honeydew production, with related negative effects on fecundity, egg viability and development (Piyaphongkul et al., 2012b).

In summary, changes in the global climate have become more interconnected, having both direct and indirect effects on ecosystem responses. Ectothermic organisms are thought to be particularly at risk from global warming since their physiological performance is directly dependent on temperature. With knowledge from this study of the thermal limits of different life cycles stages of *N. lugens*, their acclimatory ability, and effects of sub-lethal heat stress on development, fecundity and feeding activity, it is reasonable to conclude that this pest species has the potential to become even more damaging as a result of climate change, but the areas experiencing severe pest damage may change over time. Thus, extreme high temperature events may become more common in summer in tropical sub-regions and locally too stressful in some areas, with negative effects on development, reproduction and survival. By contrast, higher temperatures in other parts of the distribution may in future allow year-round residency in areas where this is currently impossible.
8.5 Future research

This study is one of the first investigations on the effects of high temperature stress on a tropical insect, using *N. lugens* as a model species. The extension of this approach to other tropical species would enable some general conclusions to be drawn. Even on the basis of the data for *N. lugens*, it is likely, however, that the upper thermal limits of other tropical insects are already overlapping current and future high temperatures, and unless the insects are able to evolve biochemical molecules that denature at higher temperatures than at present, there are likely to be areas where populations will suffer higher levels of mortality than at present, with a redrawing of distributions over time. With regard specifically to *N. lugens* it must be recognized that all of the experiments undertaken in this project were carried out under laboratory conditions, so it is difficult to extrapolate the data to field situations, not least because of the greater complexity of natural environments and the tri-trophic interactions between plants, herbivorous insects and natural enemies (Bale et al., 2002). The key challenges for future research in this area are therefore to conduct similar investigations on other tropical species, including comparisons between geographically distinct populations of widely-distributed species and with natural enemy species, and design field experiments to test the hypotheses derived from laboratory studies.
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