UNDERSTANDING MECHANISMS BEHIND THE TOXICITY OF ETHANEDINITRILE, AN ALTERNATIVE FUMIGANT TO METHYL BROMIDE

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Godwin Nutifafa Gidiglo

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Thesis Supervisors

Dr. Adriana J. Najar-Rodriguez (Plant and Food Research, Palmerston North, New Zealand)

Dr. Matthew M.K.D Hall (Department of Industry, Tourism and Trade, Darwin, Australia)

Dr. Maria Minor (Massey University, Palmerston North, New Zealand)

Professor Wang Qiao (Massey University, Palmerston North, New Zealand)

Thesis examiners

Dr. Andrea Clavijo-McCormick (Massey University, Palmerston North, New Zealand)

Professor Phil Lester (Victoria University of Wellington, Wellington, New Zealand)

Professor Vaclav Stejskal (Czech Agricultural University, Czech Republic)

Abstract

Ethanedinitrile (EDN, syn. cyanogen, EDN Fumigas®) has the potential to replace methyl bromide for the phytosanitary treatment of export timber and logs. Previous studies have shown that the toxicity of EDN differs between insect species and their life stages. For example, adults of the golden-haired bark beetle, *Hylurgus ligniperda* (Fabricius) (Coleoptera: Curculionidae) are highly tolerant to EDN, while those of the burnt pine longhorn beetle, *Arhopalus ferus* (Mulsant) (Coleoptera: Cerambycidae) are highly susceptible, implying that the mode of action might be species-specific. To understand the mechanisms behind the toxicity of the fumigant to insects, I examined the anatomy of the cuticle and spiracles of adults of both beetles. I found that *H. ligniperda* possessed smaller total spiracle area, suggesting the possibility of less EDN uptake; and more cuticle layers, suggesting the possibility of delayed penetration of the fumigant. These morphological features might limit the uptake of EDN and the related toxic effect. Further studies have shown that the main route of entry for EDN into the insect body was through the spiracles for *A. ferus*, and through the cuticle for *H. ligniperda*. The fumigant caused a reduction in walking activity, narcosis, and paralysis in both species. Reductions in walking activities and narcosis occurred earlier in *H. ligniperda*, suggesting that this species may be able to quickly decrease metabolism and respiration rates in response to EDN, which could minimize uptake and toxic effect of the fumigant. Using attenuated total reflection - fourier transformed infrared (ATR - FTIR) spectroscopy, I identified higher cyanide (CN^-) peaks in the insect ganglia in adult *A. ferus*, confirming the higher susceptibility of this species to EDN. This study has contributed to the knowledge of mechanisms behind the variable toxicity of EDN to adult *A. ferus* and *H. ligniperda*. The findings will provide a platform for future studies of the mode of action of EDN against insect pests.

Acknowledgements

I will give thanks to you with all my heart, oh Lord. I will remind you about all your wonderful deeds upon my life (Psalm 9:1). There is none to be compared unto thee (Jeremiah 10:6). Indeed, I have felt your intervention all these while. The unflinching support and blessings you offered me were unmatched. I say, “akpe na wo”.

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List of Abbreviations

Abbreviation	Meaning
EDN	Ethanedinitrile
DDT	Dichlorodiphenyltrichloroethane
NZD	New Zealand dollars
MB	Methyl bromide
MPI	Ministry of Primary Industries
NZ	New Zealand
GDP	Gross Domestic Product
CSIRO	Commonwealth Scientific and Industrial Research Organisation
LD	Lethal Dose
HCN	Hydrogen cyanide
CN-	Cyanide ion
EPA	Environmental Protection Authority
LC	Lethal Concentration
CH3CN	Methyl Cyanide
CNS	Central Nervous System
HOCN	Cyanic acid
PVC	Polyvinyl chloride
USA	United States of America
CT	Concentration-Time
TLV	Threshold Limit Value
TEM	Transmission Electron Microscope
LM	Light Microscope
GLM	Generalised Linear Model
LSD	Least Significant Difference
ANOVA	Analysis of Variance

CHAPTER 1 General Introduction

1.1 Background

In New Zealand, the sale of forest wood products is the third-largest export earner after meat and dairy and was valued at approximately NZD 6.7 billion in 2019 (MPI 2020). Over the years, there has been a growing trend in the reliance of New Zealand forest growers on export logs, with great expectations for upcoming years. The sustainability of this forest wood product trade largely depends on the effective handling of export-bound commodities. Therefore, to control infestations from quarantine insects and meet the phytosanitary requirements of importing countries, exportable forest wood products from New Zealand are fumigated either with methyl bromide (MB) or phosphine or debarked (Cross 1991). Since MB is ozone-depleting (Thomas 1996), the New Zealand Environmental Protection Authority (EPA) advised that all the fumigant remaining under the tarpaulin after fumigation needs to be recaptured or destroyed by April 2021 (EPA 2020). Hence, efforts are being made to find alternatives.

Ethanedinitrile (EDN) is approved in New Zealand as a treatment option for controlling adults of the burnt pine longhorn beetle, *Arhopalus ferus* (Mulsant) (Coleoptera: Cerambycidae) on wood products exported to Australia. EDN is a new fumigant which is an ozone-safe alternative to MB (Pranamornkith et al. 2014c). As part of efforts to evaluate the environmental and ecotoxicology fate for the risks associated with EDN exposure, trials have confirmed that during large-scale fumigations, the fumigant breaks down rapidly within the atmosphere as soon as the tarpaulin is removed from the log stacks for ventilation (EPA 2018). Furthermore, 24 hr after fumigation, very insignificant amount of EDN is released from the logs and only 1 % of the applied fumigant remains in the atmosphere. Therefore, plants, aquatic or terrestrial lives are not directly at risk of exposure to the fumigant. During log fumigations, the fumigant is rapidly absorbed by the logs. Inside the logs, EDN breaks down quickly to form cyanide which kills the pest species present.

Although EDN has been approved for this use, it is not currently registered in New Zealand. However, an application for permit for its use has been logged with the EPA (APP202804), but this process is ongoing (EPA 2020), pending assessement of its environmental and human impacts. EDN has a broad spectrum of efficacy (Ducom 2006) and is also effective in treating

soil, insect pests, weeds and diseases (Hanson & Shrestha 2006).

1.2 Importance and relevance of this research

Given that EDN is currently regarded as a promising alternative to MB for the treatment of timber and logs (Brash et al. 2013; Pranamornkith et al. 2014d; Ren et al. 2006), fundamental research is required to understand how the fumigant kills insects. An understanding of the underlying mechanisms behind the toxicity of EDN to insects, particularly those of concern to New Zealand's forestry sector, is important in facilitating the design of effective fumigation protocols, for the control of the target species and the achievement of market access of export logs and wood products.

The efficacy of EDN against wood-boring insects has been reported in recent years. For example, all life stages of *A. ferus* were extremely susceptible to the fumigant (Najar-Rodriguez et al. 2015a) while adults of the golden-haired bark beetle, *Hylurgus ligniperda* (Fabricius) (Coleoptera: Curculionidae), were highly tolerant, even after prolonged hours of exposure (Najar-Rodriguez et al. unpublished data). The reasons behind differences in tolerance between the two forest insects are still unclear. Differences in cuticle thicknesses were cited as reasons why larvae of the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) were resistant to *B*-cypermethrin (Lin et al. 2012), with the cuticle of resistant strains being thicker and having more chitin layers than that from the susceptible ones. Furthermore, differences in the mode of action of some insecticide classes against four cockroach species: *Blatella germanica* (L), *Periplaneta americana* (L), *Blatta orientalis* (Stoll) and *Supella longipalpa* (Serv.) (Dictyoptera: Blattidae) were associated with differences in the structure and function of the tracheal system of the insects (Chaudhari 2016). As the uptake of EDN through the tracheal system or the insect cuticle is possible and the number and size of spiracles and their locations along the body of insects may vary across species (Keilin 1944), I conducted studies on the anatomy of cuticle and spiracles of *A. ferus* and *H. ligniperda* adults to determine if those traits contribute to their differences in tolerance to EDN. I also tested the main routes of entry for EDN into the insects to investigate the underlying mechanisms behind species-specific differences in tolerance to the fumigant, as reported in the literature.

Differences in tolerance to EDN across insect species may also be associated with the possession of unique adaptations. For instance, insects have shown to be capable of withstanding insecticide exposure through physiological adaptations or behavioural modifications, by avoiding or minimizing their encounter with different toxic compounds (Jallow & Hoy 2005; Lee et al. 2007b). Though, such behavioural modifications may be beneficial to the insects, they could compromise fumigant efficacy (Bayley & Dell'Omo 2002; Cox et al. 1997; Moore et al. 1989). According to Cotton (1932), the rate of respiratory metabolism affects the susceptibility of insects to fumigants. Reduced walking activity lowered the respiration rates of the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (May 1989) and *Exochomus quadripustulatus* L. (Coleoptera: Coccinellidae) (Fytizas & Katsoyannos 1979), of target insects and that could minimize their uptake of EDN. Therefore, to determine the effectiveness of insecticides on insect pests, accurate assessment of both behavioural and physiological effects on the overall efficacy of the toxic compound is important (Biondi et al. 2012). Behavioural responses may also reflect the mode of action and the final target sites for pesticides (Haynes 1988). Even though no records of EDN-induced behavioural adaptations in insects are found in literature, some studies have measured behavioural responses of insects to other fumigants. For instance, phosphine exposure was found to decrease walking activity in the lesser grain borer, *Rhyzopertha dominica* (Fabricius) (Coleoptera: Bostrichidae) (Pimentel et al. 2012). Also, resistant and susceptible strains of the rice weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae) exhibited different rates of flight take-off when exposed to deltamethrin (Guedes et al. 2009b). Understanding the behavioural effects of EDN in this context would thus help understand its mode of action against target insects.

EDN is soluble in water and is thought to break down to hydrogen cyanide (HCN) and other minor components (e.g. ammonia and CO_2) (Brash et al. 2013; O'Brien et al. 1999). However, the theoretical breakdown of EDN to HCN in treated space may not occur, as Park et al. (2014) and Hall et al. (2018a) did not find the breakdown product in fumigated logs. For insects, the efficacy of EDN is believed to involve the toxic action of cyanide ions (CN^-), which bind to the cytochrome c oxidase (COX) enzyme in the cell mitochondria (Hamel 2011), causing a reduction in oxygen supply to insect tissues, especially those with high oxygen demand and eventually killing the insects (Anseeuw et al. 2013). Therefore, detection of HCN or CN^- and other break-down products inside the insect bodies may be essential in the assessment of the overall toxicity

of the fumigant. Further investigations on the final target organ(s) of each breakdown product is also an important requirement in defining the mode of action of EDN against insects.

1.3 Aims and objectives of this study

This research aimed to investigate the underlying mechanisms behind the toxicity of EDN to two of New Zealand's key forest pests: *A. ferus* and *H. ligniperda*, with five objectives:

1) To review previous research efforts and acquire current knowledge on both beetle species and EDN (Chapter Two),
2) To determine the main point(s) of entry for EDN into the bodies of target insects (Chapter Three),
3) To investigate the effects of EDN fumigation on the behaviour of target insects (Chapter Four),
4) To identify target organ (s) and quantify the breakdown product (s) of the fumigant in the insect bodies (Chapter Five), and
5) To propose a potential mode of action of EDN by linking all the results of this research and to suggest further research that could contribute to the understanding of the mechanisms underlying the toxicity of the fumigant against target insects as derived from this research (Chapter Six).

CHAPTER 2 Literature Review

2.1 Introduction

The New Zealand economy relies on the export of *Pinus radiata* (D.Don) timber and log products, as the third-largest sector of revenue generation (MPI 2019). The export markets require effective control of quarantine insects that accompany wood products (Najar-Rodriguez et al. 2015a). Methyl bromide fumigation is currently the most effective approach to quarantine and pre-shipment (QPS) treatments for international markets (MPI 2019). However, because MB is an ozone-depleting compound (Earth 1995), its use in New Zealand henceforth, will require recapture or destruction to control atmospheric emissions by April, 2021 (EPA 2020). Ethanedinitrile has shown great promise as a viable alternative (Pranamornkith et al. 2014d; Park et al. 2014; Ren et al. 2011). However, previous studies have shown that the fumigant is selectively toxic to insects (Hooper et al. 2003; Najar-Rodriguez et al. 2015b; Pranamornkith et al. 2014c). Therefore, research into the mode of action against insects is required.

For this thesis, attention was focused on two of New Zealand's key forest beetles: *H. ligniperda* and *Arhopalus ferus*. In previous efficacy studies, these species have shown contrasting levels of tolerance to EDN. The current literature review included the above-mentioned studies plus general aspects of EDN as a fumigant and its chemical properties, as well as its efficacy against other insect pests. Furthermore, the mode of action of fumigants involving their point(s) of entry into insects, factors that affect their uptake and information on HCN were reviewed. Where appropriate, references were made to other fumigants that have shown various mechanisms of toxicity against insect pests. Lastly, the effect of fumigation on insect behaviour was also discussed.

2.2 The burnt pine longhorn beetle, *Arhopalus ferus* (Mulsant)

Longhorn beetles are examples of the most frequently established groups of invasive forest insects across the world (Eyre & Haack 2017). Within the group is *A. ferus*, which is one of the most important insects of market access interest in New Zealand (Allison et al. 2004).

2.2.1 Classification and distribution

The *Arhopalus* genus was first described by Serville in 1834 (López et al. 2008). It has about 25 species described worldwide (Wang & Leschen 2003) and belongs to the subfamily Spondylidinae of the family, Cerambycidae (Sopow et al. 2015). The Cerambycidae family is one of the largest families of Coleopterans with over five thousand three hundred genera and thirty six thousand three hundred species discovered worldwide (Tavakilian & Chevillotte 2015). Cerambycid insects are believed to have inhabited the world's main biogeographic regions via international trade and globalisation (Brockerhoff et al. 2017). The largest subfamilies (Lamiinae, Cerambycinae and Prioninae) are usually found in the tropical and sub-tropical zones of the world (Slipinski & Escalona 2013). In New Zealand, one hundred and eighty eight species from the Cerambycidae were reported (Sopow & Bain 2017). Of the number, 111 species belong to the Lamiinae, seventy five from the Cerambycinae, one from the Prioninae, and one from the Spondylidinae (Macfarlane et al; Sopow et al. 2015).

A. ferus is distributed throughout Europe, northern Asia (except Japan), and North Africa (Gobbi et al. 2012). The insect was accidentally introduced into New Zealand in the mid-1950s (Hosking & Bain 1977) but was first reported in Australasia by Milligan (1970). The first detection dates back to 1963 in a fire-burnt *P. radiata* plantation at Mamaranui, North of Auckland (Kerr 2010). Currently, *A. ferus* is widely spread throughout New Zealand (Brockerhoff et al. 2006a & Pawson et al. 2009).

2.2.2 Host plants and importance

A. ferus is mainly associated with coniferous plants, especially *Pinus* species and the Norway spruce, *Picea abies* (Bense 1995). In Europe, *P. abies* is the main host (Brockerhoff & Hosking 2001). In New Zealand, *A. ferus* can be found associated with at least eight *Pinus* species

(Hosking & Bain 1977) sometimes, in Douglas fir, *Pseudotsuga menziesii* (Mirb.) and the European larch, *Larix decidua* (Mill.) (Sopow et al. 2015). The insect can also be found on export-bound *P. radiata* logs (van Epenhuijsen et al. 2012).

Even though *A. ferus* does not cause serious damage to forests in New Zealand, the insect rapidly attacks fire-burnt forests, discarded waste wood and reduces the available time for harvesting freshly-felled host trees (Linsley 1962; Hosking & Bain 1977; Bradbury 1998; Duffy 1968). Therefore, in forest areas that have high frequency of fire outbreaks, *A. ferus* may become an important pest. The insects are also attracted to odour from burnt pine and other host volatiles like ethanol and α-pinene (Brockerhoff et al. 2006a). The appearance of large numbers of *A. ferus* at wood processing or storage sites and sawmills may be explained by the emission of large quantities of attractive volatiles from the sources (Suckling et al. 2001). These beetles are also attracted to lights (Pawson et al. 2009; Wang & Leschen 2003) and are active at dusk through the early evening (van Epenhuijsen et al. 2012).

During their flight season, which is usually in November through March in New Zealand, adults can be seen flying in the evenings. Therefore, insect collection can be done in the evenings and early mornings. The rapid deterioration of wood may occur when larvae enter the sapwood, as a result of concentrated egg-laying by adults (Santolamazza-Carbone et al. 2011). Adults are also vectors of sapstain and decay fungi (e.g., *Ophiostoma* spp.) that cause the devaluation of salvaged wood (McCarthy et al. 2010; Bradbury 1998). The presence of adult *A. ferus* on sawn timber and their attraction to wood processing sites and sawmills, poses serious quarantine concerns for timber exports (Suckling et al. 2001). As a result, importing countries require stringent phytosanitary measures (Pranamornkith et al. 2014a). The frequent use of timber packets by adult *A. ferus* as a daytime shelter may result in the transportation of infected timber packets or of *A. ferus* adults themselves as hitchhikers (Pawson et al. 2009).

2.2.3 Morphology and life cycle

Adult *A. ferus* (Fig. 2.1, 2.2 A) are brown to black and about 8 to 30 mm in length (Kerr 2010; Wang & Leschen 2003). The body is elongated and broadly oval. The head (Fig. 2.2 B, E) projects forward in an oblique form, with visible mouthparts. The prothorax has no spines (Fig. 2.2 C). The elytra have four longitudinal ridges with parallel, slightly narrow sides (Fig. 2.2 D).

Female adults (Fig. 2.1 B) are generally dark brown to black while males (Fig. 2.1A) are usually lighter brown. Males are typically smaller than females (van Epenhuijsen et al. 2012). The antennae of males which are half to three-quarters the lengths of their bodies are longer than those of females (Fig. 2.1). However, the most accurate criterion of assessment is the examination of the external genitalia. As shown in Fig. 2.1 B, the last abdominal segment of females is narrow and rounded, extending beyond the elytra.

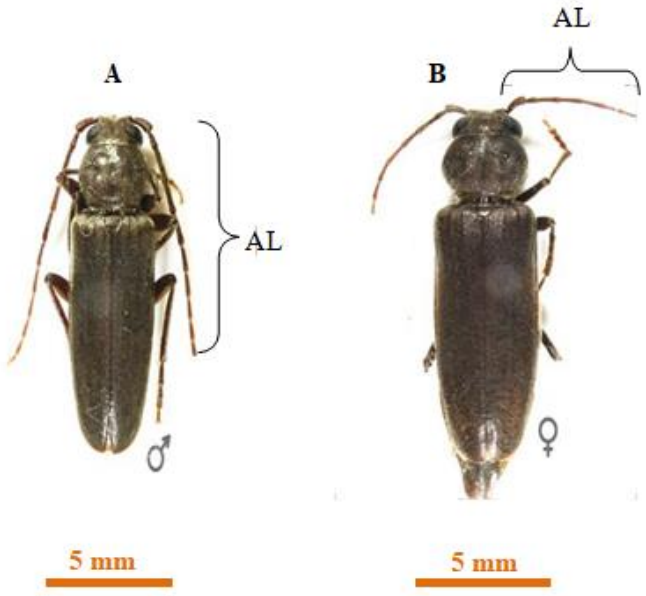

Figure 2.1 *Arhopalus ferus* adults: (A) male; (B) female. AL is the antennal length. Images were taken using LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

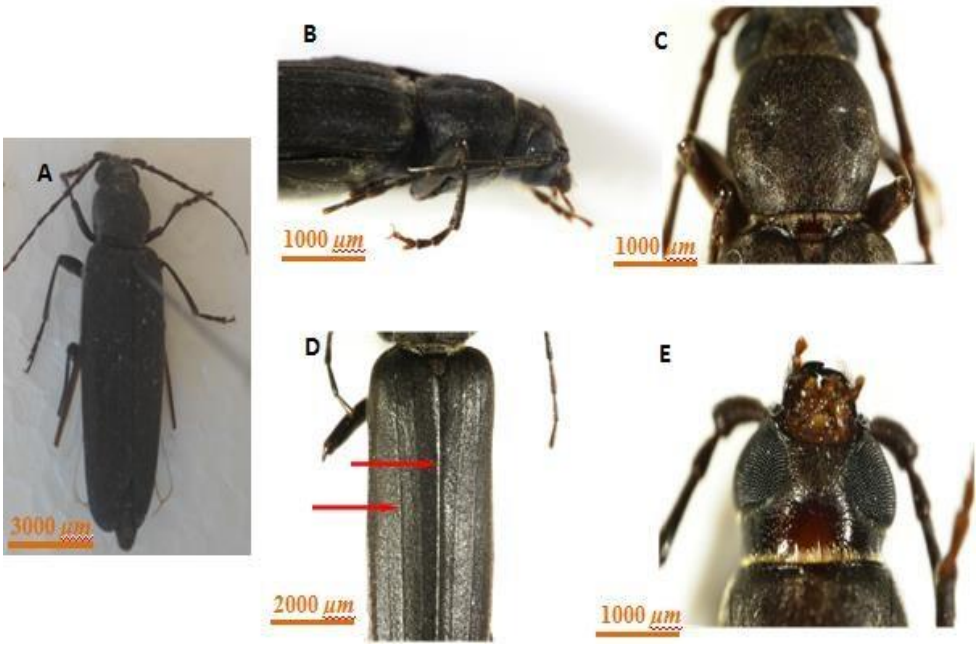

Figure 2.2 Different body parts of *Arhopalus ferus*. (A) the whole body of the adult insect, (A) lateral view of the head, thorax and part of the abdomen, (C) dorsal view of pronotum, (D) dorsal view of the elytra showing some longitudinal ridges (arrowed), and (E) ventral view of the head. Images were taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

A. ferus life cycle includes egg, larva, pupa and adult life stages (Fig. 2.3). A complete life cycle in Europe is between three to four years, during which time adults can be seen from the beginning of summer to the beginning of autumn (Bense 1995; Duffy 1968). In New Zealand, the beetles take one to two years to complete their life cycle (Hosking & Bain 1977; Sopow et al. 2015), and the flight season occurs from November to May, with a peak in January and February (Brockerhoff & Hosking 2001). Females lay up to one thousand eggs, which are opaque, white and cigar-shaped (Fig. 2.3), measuring approximately 0.5 by 1.8 mm. The eggs are usually laid in groups of five to fifty in bark crevices of logs and dying or dead trees within 24 hr after a fire outbreak (Sopow et al. 2015). During heavy infestations, egg groups are evenly spaced (about 15 cm apart) across the host surface. In unburned host materials, spacing is often wider. The egg incubation period varies from 9 to 12 days at 20 – 22 °C (Romo et al. 2018).

Of all life stages, the larval stage has the longest duration of up to two years depending on prevailing temperatures. Larvae appear cylindrical, flattened, creamy white with legs, mandibles and a pair of blackish projections at the tip of the last abdominal segment (Fig. 2.3). They are very active when removed from their tunnels (Kerr 2010). After hatching, crowded larvae may enter the inner phloem or cambium when there is no competition. As the young larvae feed on the inner phloem, the older ones tunnel into the outer sapwood (Hosking 1978) and may pose a major threat to wood quality. The tunnels appear oval in cross-section and are around 12 mm wide. When larvae mature, they create pupal chambers with exit holes on the surface of the bark (Hosking 1978).

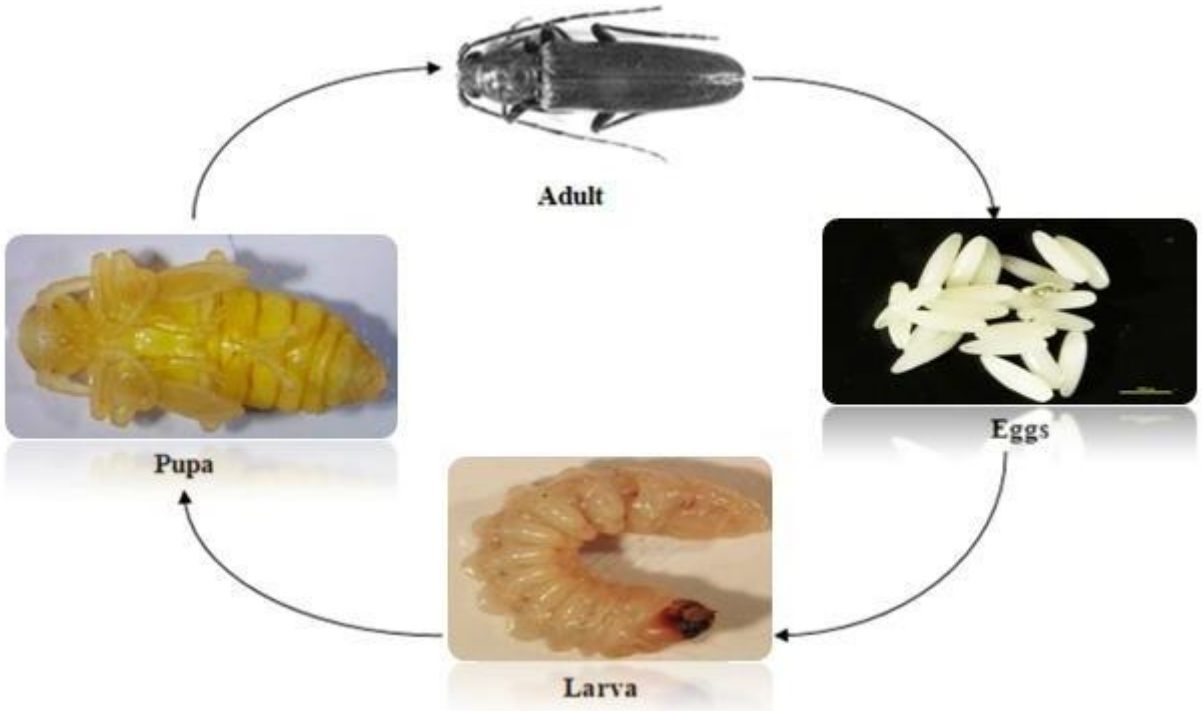

Figure 2.3 Life cycle showing eggs, larva, pupa and adult stages of *Arhopalus ferus*. Images were taken using LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

2.3 The golden-haired pine bark beetle, *Hylurgus ligniperda* (Fabricius)

The scientific name for the golden-haired pine bark beetle is *Hylurgus ligniperda* (Tribe 1991). Earlier names for this insect were *Hylesinus ligniperda, Bostrichus elongatus, Hylesinus flavipes, Hylurgus elongatus, Hylurgus flavipes* and *Hylurgus longulus* (Brockerhoff et al. 2003). *H. ligniperda* is an introduced forest insect in New Zealand and currently comprises more than 90 % of all bark beetle populations in the country's pine forests (Bain 1977; Reay et al. 2001; Brockerhoff et al. 2006b).

2.3.1 Classification and distribution

The original name for *H. ligniperda* (Coleoptera: Curculionidae: Scolytinae), as described by Fabricius in 1787 was *Bostrichus ligniperda* (Park et al. 2017). The insect is native to Europe but became an introduced species to Argentina, Australia, Brazil, Chile, Uruguay, South Africa, Paraguay, USA, Sri Lanka, Japan and New Zealand through trade (Haack 2006). The introduction of *H. ligniperda* into New Zealand is believed to be through the importation of sawn timber from South Australia. In New Zealand, the insect was first recorded near Whitford (a town in south-east Auckland) in 1974 (Brockerhoff 2009). According to Romo et al. (2015), it is the most common bark beetle in New Zealand and can be found in forest regions in the North and South Islands (Chase et al. 2017).

2.3.2 Host plants and importance

The hosts of *H. ligniperda* are species in the family Pinaceae such as *Pinus*, *Picea*, *Pseudotsuga* (Douglas fir), *Abies* (true fir) and *Larix* (larch) species (Cosner 2013). In New Zealand, the adults breed mainly in recently-felled *P. radiata* stumps and logs (Bain 1977). The insects may cause economic damage to wood by introducing sapstain fungi (Reay 2000; Harrington 1988; Zhou et al. 2004) or *Ophiostomatales* sp. (Harrington 198l; Harrington 1993; Harrington 2005; Paine 1997; Six 2003). The fungal pathogens have been isolated from populations of *H. ligniperda* in South Africa (Zhou et al. 2001), Chile (Zhou et al. 2004), Spain (Romon et al. 2007) and New Zealand (Ray et al. 2006). Generally, *H. ligniperda* is regarded as a secondary pest because it does not aggressively kill trees (Haack 2006), even though it may sometimes attack injured or stressed trees (Neumann 1987). Adults can cause severe damage by creating

brood galleries and feeding tunnels beneath the bark of harvested logs (Fig. 2.4) and eventually introduce decay fungi that degrade timber quality if the processing is not done at the right time (Kerr et al. 2017). In South Africa, *H. ligniperda* are usually root-dwellers but can enter and infest host plants directly through the soil (Tribe 1992). In Spain and Chile, adults surrounded and killed 1-2 years old seedlings of *P. radiata* during the winter (Reay & Walsh 2001). However, such incidence had neither been reported in New Zealand nor from other parts of the world.

Figure 2.4 Brood galleries created by *Hylurgus ligniperda* adults beneath the bark of harvested pine logs. Images were taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

2.3.3 Morphology and life cycle

The adult *H. ligniperda* (Fig. 2.5 A) is 4 to 6 mm long and 2 mm wide, with a dark-brown, cylindrical body (Bain 1977). The insect is referred to as "golden-haired" because of the appearance of red to yellowish hairs (Fig. 2.5 B-D) on the body (Lee et al. 2007a), which are more pronounced at the posterior part (Fig. 2.5 D). The antennae of *H. ligniperda* (Fig. 2.5 E) appear reddish-brown. The pronotum (Fig. 2.5 C) possesses punctures.

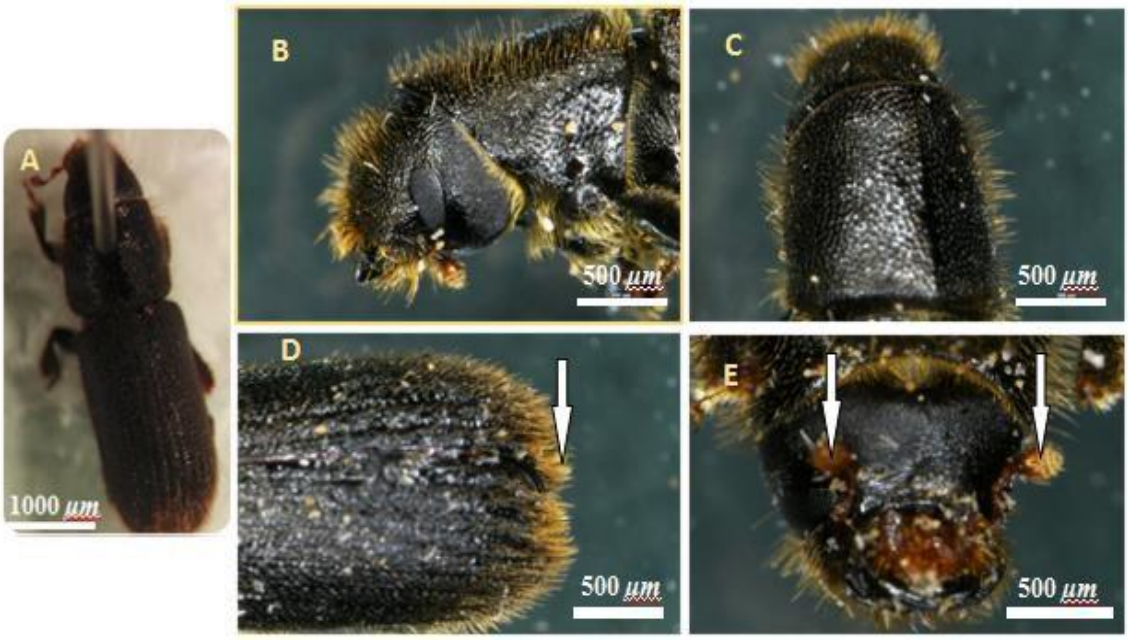

Figure 2.5 Different body parts of adult *Hylurgus ligniperda*; (A) adult insect, (B) lateral view of the head, (C) dorsal view of pronotum, (D) apex of elytra, showing clustered red to yellowish hairs (arrow), and (E) ventral view of the head, showing antennae (arrows) and mouthparts. Images were taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

The males of *H. ligniperda* can be distinguished from the females because they produce an acoustically distinguised sound when distressed (Bedoya et al. 2019; Mausel et al. 2006); the females do not make distress calls (Liu et al. 2008). Morphological characteristics of the posterior two abdominal tergites can also be used to differentiate males from females (Bedoya et al. 2019; Liu et al. 2008). The posterior margin of the sixth tergite of the male insects is sclerotized and has more bends (Fig. 2.6 C). Also, the seventh tergite is sealed by the sixth tergite in females (Fig. 2.6 D), and the length of the sixth abdominal tergite of the female is equal to the combined length of the sixth and seventh abdominal tergites of the male (Liu et al. 2008).

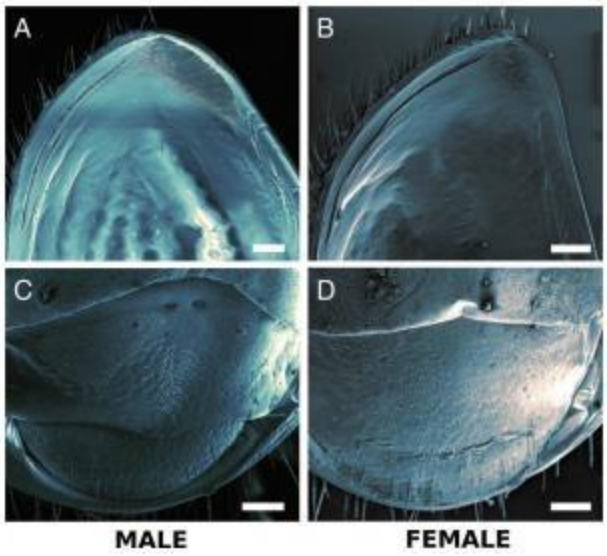

Figure 2.6 Gender differences in *ligniperda*. (A) the ventral part of the left elytron in a male, (B) ventral part of the left elytron in a female, (C) dorsal part of the last three posterior tergites beneath elytra and wings in a male, and (D) dorsal part of the last three posterior tergites beneath elytra and wings in a female. The seventh abdominal tergite in females is veiled by the sixth tergite, with a size comparable with the combined size of the sixth and seventh tergites in males. Scale bars = 100 *μm* (With permission from Bedoya L. Carol, the corresponding author). Images were taken using a SEM microscope, Plant and Food Research Institute, Auckland, New Zealand.

H. ligniperda has four larval stages with at least one generation a year (Brockerhoff et al. 2003). In southern Europe, adult flight season occurs from March to April (Grune 1979). In the south-east of France, two generations can be observed in a year (Fabre & Carle 1974). In South Africa, peak flight activity is between April and May (Tribe 1991). In Chile, up to four generations can be experienced per year (Gómez et al. 2017). In New Zealand, the life cycle of *H. ligniperda* (Fig. 2.7) usually takes ten to eleven weeks (Bain 1977), with two generations in cooler regions and between two to three generations in warmer regions in a year.

The female *H. ligniperda* constructs egg galleries (Fig. 2.4) with a short entry tunnel into the inner bark. She widens the nuptial chamber and selects a male for mating. Single egg galleries are usually 10 to 20 cm long from the nuptial chamber along the wood grains. During the construction of gallery, females usually dig at the end of the tunnel. At this time, the males assist in either removing frass from the tunnel or making short feeding tunnels that connect to the egg galleries (Browne 1968). The female lays up to five hundred eggs (Lee et al. 2007b) which

appear ovoid and pearly white (Clare & George 2016a). The egg-laying period lasts for up to six weeks (Liu et al. 2007), during which the females feed.

Eggs hatch in about two weeks during summer (Kimoto & Duthie-Holt 2004). The larval gallery usually appears at right angles to the egg gallery and does not follow a definite pattern of arrangement. The body wall of a small larva is transparent and shows the reddish gut (Bain 1977). The fully-grown larvae are legless and C-shaped (Fig. 2.7). The head of the larva is light brown, with two dark circular protuberances on the front portion of the head just above the jaws. Mature larvae are often seen closer to the nuptial chamber, followed by a medium to smaller-sized ones at successive intervals along the tunnel (Dumouchel & Palisek 2002).

Fully-grown larvae pupate at the end of their tunnels. The pupae look yellowish (Fig. 2.7) and have a duration of one to two weeks. Mature adults emerge in about two to three weeks. Newly-emerged adults feed and form spiral feeding galleries at the root collar regions of pine seedlings.

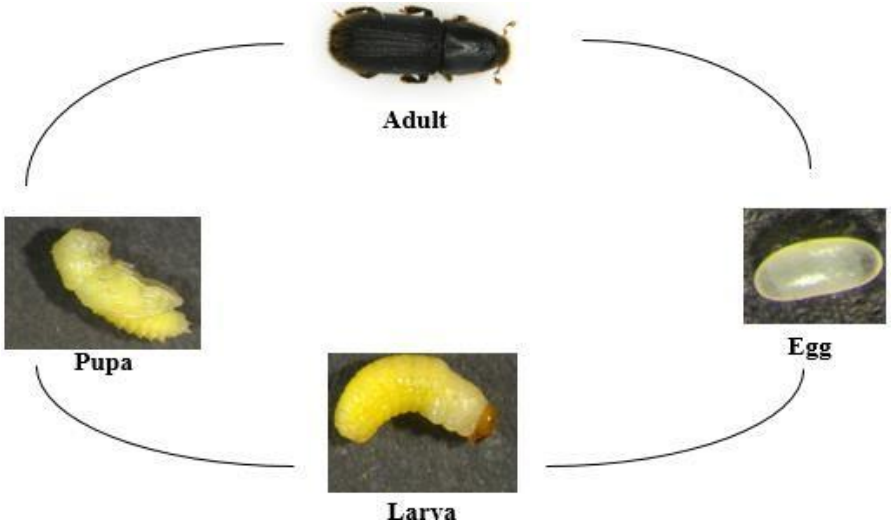

Figure 2.7 Life cycle stages of *Hylurgus ligniperda* showing a freshly-laid egg, mature larva, pupa and adult collected from stripped *Pinus radiata* logs. Images were taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

2.4 Ethanedinitrile (EDN)

Ethanedinitrile is a new fumigant with an almond-like odour and chemical formula, C_2N_2 (Emekci 2010). It has shown excellent potential as an alternative to MB in the quarantine treatment of timber and wood products (Ren et al. 2011; Ren et al. 2006; Park et al. 2014; Pranamornkith et al. 2014b). Several methods of preparing the compound have been described in the literature. However, Joseph Louis Gay-Lussac was the first to use the thermal decomposition of silver cyanide to prepare a small amount of EDN in 1815 (Fierce & Millikan 1958). The large-scale production of EDN started in mid-1916 to develop cyanogen chloride for the production of nitrocellulose (Fierce & Sandner 1960). In 1960, Fierce and Sandner developed a cheap method for its production (Bircumshaw et al. 1954; Fierce & Sandner 1960). More recently, the fumigant became useful for the nitrate fertilizer industry (Armstrong et al. 2014).

The potential of EDN as a fumigant was first discovered by the Commonwealth Scientific and Industrial Research Organisation (CSIRO) in 1996 (Waterford et al. 2008). The threshold limit value (TLV) of 10 ppm (v/v) for EDN compares favorably against that of MB (5 ppm) and phosphine (0.3 ppm) (Ren et al. 2006). TLV is the concentration of EDN in the air averaged over an 8-hr work day and a 40-hr work week to which it is believed that workers may be repeatedly exposed, day after day, for a working lifetime without adverse effects. EDN is effective at treating soil, insect pests, nematodes, weeds, fungi and diseases (Ducom 2006). In 2011, EDN was registered in Australia as a disinfestation treatment for logs and sawn timber (Pranamornkith et al. 2014d), and since then, it became available for research purposes in New Zealand (Najar-Rodriguez etal. 2015b). It was approved in 2017 as a treatment option to control adult *A. ferus* on Australia- bound wood product exports from New Zealand (MPI 2018). Currently, EDN is not registered in New Zealand. However, an application has been submitted with the New Zealand EPA (APP202804), pending approval for its use.

2.4.1 Physical and chemical properties of EDN

EDN is a colourless flammable gas at room temperature (Singh & Sharma 2015). It has freezing and boiling points of -28.0 °C and -20.7 °C, respectively (Hanselmann et al. 2012), and can be applied directly without the use of vaporisers or specialised equipment. It is heavier than air

(O'Brien et al. 1999) and can settle in containers. Additionally, the fumigant has great penetration into timber and logs (Ren et al. 1997), with high efficacy and short application time (Ryan et al. 2006). Earlier work by Ren et al. (1997) reported a significantly greater sorption rate when EDN was used to fumigate *P. menziesii*. EDN sorption can be affected by several factors commonly experienced during commercial situations. Pranamornkith et al. (2014b) evaluated the effects of dose, moisture content, end-grain sealing and load factor on sorption of EDN by sawn timber at 15 °C and reported that chamber loading significantly influenced sorption. However, sorption pattern was not affected by the fumigant. Increased moisture content and end-grain sealing both reduced sorption, but the effects were negligible and the differences in sorption patterns caused by moisture content or end-grain sealing decreased over time.

In a follow-up study, Hall et al. (2015) quantified the sorption characteristics of EDN for freshly harvested *P. radiata* logs and reported that EDN sorption was influenced by the dose applied. Considering the high sorption of EDN from the above experiments, coupled with the fact that as a fumigant, one of its routes of entry into insects may be through the respiratory system, there are doubts regarding the inhalable amount that could effectively control insects. Even though low sorption is an important characteristic of a useful fumigant (Lorraine 2014), EDN might be an exception. Brierley et al. (2019) developed and validated a method to quantify EDN, using a gas chromatograph equipped with a flame ionization detector (FID).

The active ingredients of EDN are HCN, formic acid, and carbon dioxide (CO_2). Even though EDN has a strong affinity for water and may rapidly react to form HCN (Emekci 2010) (i), which then breaks down to yield CNO and O (ii), and NO (iii), the fumigant escapes quickly because of its volatility in aqueous media. The C_2N_2 molecule has a non-polar character with high affinity to the non-polar surface of wood and wood products, so it undergoes a quick adsorption process, which is accelerated in the presence of moisture:

$$CN\text{-}CN + H_2O \longrightarrow HCNO + HCN \qquad (i)$$

In the atmosphere, EDN hydrolyses to produce HCN and cyanic acid (HOCN). The fumigant also reacts slowly with hydroxyl radicals in the atmosphere. Most of the cyanide in HCN reacts with atmospheric O_2 molecules to produce either oxidized cyanate ion or CO and NO:

$$CN + O_2 \longrightarrow CNO + O \quad \text{(ii)}$$

$$CN + O_2 \longrightarrow CO + NO \quad \text{(iii)}$$

Therefore, EDN does not deplete ozone as does MB.

During fumigation, EDN concentration was reported to have dropped with time, with the initial dose rate of 150 g/m^3 being 1.5 g/m^3 after 24 hr (Brierley et al. 2019). In the human body, EDN dissolves in aqueous solutions within the lungs. Within the lungs, the rate of hydrolysis is very fast. At that time, EDN breaks down to produce HCN, then hydrogen, cyanide and HOCN. However, exposure to EDN is not cumulative, as the fumigant does not build up in the body (EPA, 2018).

2.4.2 The efficacy of EDN against insect pests

The potential of EDN as an effective fumigant to control insects has received attention in recent years. The first reference to studies regarding EDN efficacy on wood-related insects was by CSIRO (1996). One hundred percent mortality of three dry wood termite species (Isoptera: Rhinotermitidae) was achieved using concentrations ranging from 0.43 g/m^3 to 1.74 g/m^3 for 24 hr at 30 °C. However, only 10 to 25 small termite workers were used in this research. Wright (2000) repeated the above experiments and provided the same results, with evidence to support the fact that EDN had potential use against insects. In a research review, Viljoen and Ren (2001) gave reasons regarding EDN fumigant as a potential timber treatment option but provided no data to support their ideas.

Ren et al. (2003) investigated the efficacy of EDN against the Asian long-horned beetle larvae, *Anoplophora glabripennis* (Motchulsky) (Coleoptera: Cerambycidae) from infested logs and reported that the concentration-time (CT) products over the following range of temperatures: 4.4, 10.1, 15.6, and 20.1 °C decreased with increasing temperature, for both 3 hr or 6-hr fumigation durations. The CT products varied with exposure time for different temperatures. Although EDN was efficient in controlling *A. glabripennis* larvae, the initial concentrations and the number of replicates used in the research were not provided. EDN efficacy was also comparatively greater than MB at 4 °C but that was not stated in the results, either. Hooper et al. (2003) investigated a

broad-range spectrum of EDN toxicity to six species of stored-product insects and reported that the fumigant was not very efficient on adults of *Sitophilus* spp., even though effective control of the egg stage was achieved by a 5-day exposure of 13.7 g/m^3 or 27.4 g/m^3. Additionally, both gaseous and aqueous forms of EDN were toxic to the tested insects, and the toxicity to all tested insects increased with increasing relative humidity and the concentration of CO_2. The results suggest that EDN has great potential as an alternative fumigant for the control of a variety of stored-grain insects. However, the reasons why *Sitophilus* adults were not controlled after a long period of EDN exposure were unknown.

Cho et al. (2011) reported the results of two different tests on the efficacy of EDN against nymphal and adult stages of the Japanese termite, *Reticulitermes speratus* (Kolbe) (Isoptera: Rhinotermitidae) and the yellow minute pine bark beetle, *Cryphalus fulvus* Nishima (Coleoptera: Scolytidae) larvae and adults. Insects were fumigated using 50 g/m^3 or 70 g/m^3 of EDN for 6 hr at 22 °C or 5.0 °C and 24 hr at 4.1 °C or 6.1 °C in metal frame chambers, covered with Polyvinyl chloride (PVC) tarpaulin. In a different set of experiments, 48 g/m^3 of MB was used to fumigate the above-mentioned insects for 24 hr. The two results showed that EDN was as effective as MB and has the potential to control insect-infested logs.

Initial results in New Zealand also showed that EDN has potential as a MB alternative for the disinfestation treatment of export logs. For example, Pranamornkith et al. (2014d) studied the efficacy of EDN against *A. ferus* adults. A lethal dose of 12.6 g/m^3 was used to fumigate the insects for 3 hr. Najar-Rodriguez et al. (2015b) compared the toxicity of EDN in the laboratory to that of reduced rates of MB, using different life stages of *A. ferus*. Naked insects were fumigated with MB at 10 °C and 20 °C for 4 hr or with EDN at the same temperatures for 3 hr. The mortalities achieved showed that EDN was highly toxic to all *A. ferus* life stages tested, agreeing to previous reports by Pranamornkith et al. (2014c). The most susceptible life stage to EDN at both 10 °C and 20 °C was eggs, but no significant differences were found between the larval and the adult life stages. However, further research was needed to unravel the reasons behind the differential response across life stages of *A. ferus* to EDN. In other research by Najar-Rodriguez et al. (Unpublished data), adult, larvae and pupae of *H. ligniperda* were found to be highly tolerant to EDN.

The results outlined above suggest that even though EDN is toxic to a wide range of insects, its

mode of action might be species-specific. Therefore, in-depth knowledge of the mechanism of toxicity against target species is needed.

2.5 Insecticide mode of entry into insects

Even though many studies have investigated the mode of entry of insecticides into insects, the most widely reported ones are penetration through the cuticle, mouthparts and/or spiracles (Gerolt 1983). However, the efficacy of an insecticide largely depends on its ability to penetrate the body of insects and be able to reach a target site (Richards & Cutkomp 1946). In the case of EDN, the exact mode of entry into insects is not clearly understood.

2.5.1 The insect spiracle as insecticide route of entry

Most insects inhale gaseous substances through the tracheal system that connects to the outside environment through spiracles (Chapman & Chapman 1998). Therefore, insect spiracles are capable of providing gaseous toxic chemicals, including fumigants, a direct route of entry into the insect bodies. Spiracles (Fig. 2.8) are openings found on the thorax and abdomen of most insects (Sukontason et al. 2006). The closing and opening mechanisms of spiracles are controlled by valves and muscles (Mellanby 1934; Ramsay 1935; Miller 1962). Spiracular valves are regulated by loops that open spiracles in response to high internal CO_2 pressure or low O_2 pressure, but close in response to high O_2 pressure (Förster 2010; Heinrich 2015).

Even though transport of gases within the tracheal system of insects may take place via passive diffusion (Weis-Fogh 1964), regulation of gas exchange through active ventilation (Sláma 1988) and spiracular valves along the lateral sides of the body are also possible. Heinrich et al. (2013) monitored spiracle valve activity and the movement of respiratory gases in the hissing cockroach, *Gromphadorhina portentosa* (Schaum) (Dictyoptera: Blaberidae) and observed that abdominal spiracles of the insects opened and closed at the same time during ventilation. It was also observed that the thoracic spiracles aided inhalation of air whilst exhalation occured through the abdominal spiracles. Kaars (1981) attributed the airflow from the anterior to posterior parts of *G. portentosa* and the discoid cockroach, *Blaberus discoidalis* Serville (Dictyoptera: Blaberidae) insect bodies to the coordinated action of spiracles.

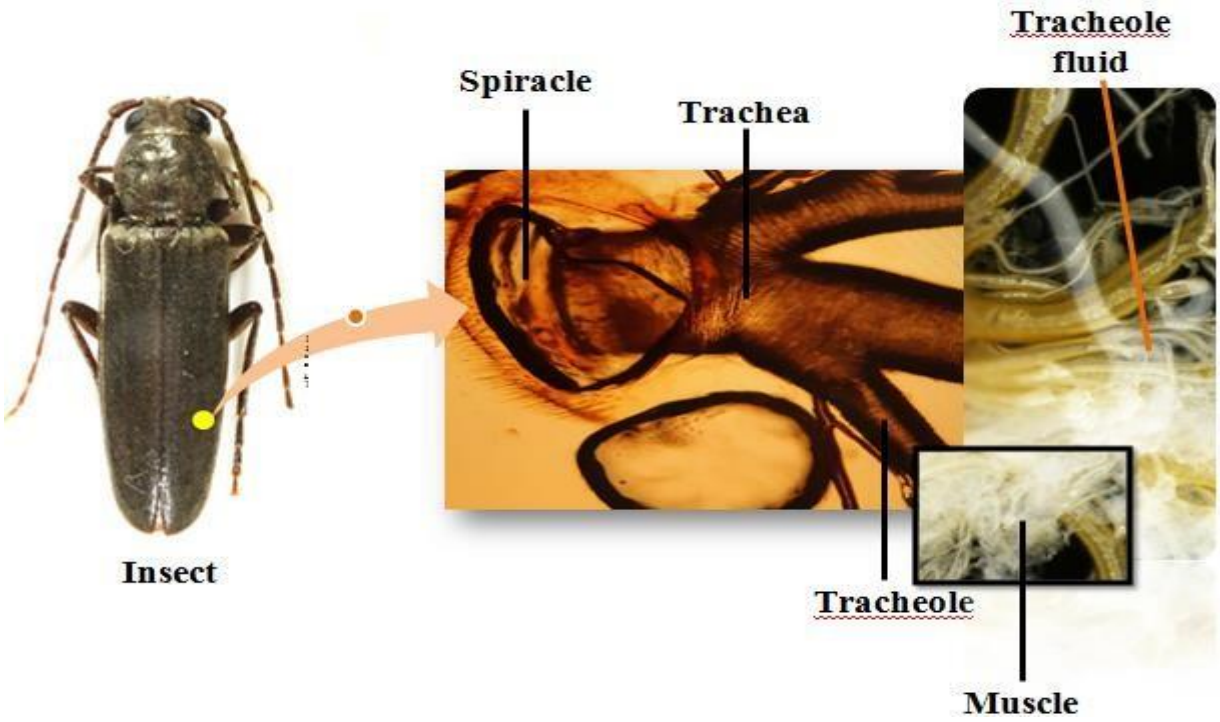

Figure 2.8 Structure of an insect tracheal system. Images were taken using LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

Huber (1974) suggested that spiracles on the ventral or dorsal mesothorax could be a primary target for pyrethroids because they allowed rapid entry into insect bodies and provided the quickest route to the central nervous system (CNS). Gerolt (1983) also observed that spiracles are the fastest and shortest route to the CNS of insects. Sugiura et al. (2008) identified the mesothoracic spiracles of *B. germanica* as one of the most effective entry routes for directly applied aerosol-based pyrethroids and suggested that the knockdown effect on the insects was caused by the compound's entry through the mesothoracic spiracles, and the eventual penetration through the inner walls of the mesothoracic tracheae. Sumita et al. (2016) also investigated the mode of entry of pyrethroids into adult housefly, *Musca domestica* L. (Diptera: Muscidae). Their results suggest that the rate of pyrethroid uptake through the spiracles decreased due to the blocking of the mesothoracic spiracles. However, only the mesothoracic spiracles were blocked in this research and the role of other spiracles was not explored. In addition, species-specific differences in spiracle morphology and/or structure may affect how a chemical enters insects

through the spiracles. In order to ascertain whether spiracles are the main routes of insecticide entry into insects, it would be helpful to explore the contribution of other spiracles as well.

2.5.1.1 Factors that affect the uptake of insecticides through spiracles

Several studies have examined the influence of CO_2 on the toxicity of fumigants. Hazelhoff (1928) reported that CO_2 enabled the penetration of fumigants by keeping the spiracles open. CO_2 gas is a respiratory stimulant in insects (Case 1957). Jones (1938) showed that CO_2 enhanced the toxicity of several fumigants against the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Sitophilus spp*. and the Khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) (Annis 1987). Carbon dioxide kept the spiracles of insects open and ensured the entry of HCN (Aliniazee & Lindgren 1969). More recently, Leelaja et al. (2007) demonstrated that CO_2 enhanced the toxicity of allyl acetate to six stored-product grain beetles. Sub-lethal concentrations of CO_2 also improved the toxicity of MB and HCN to the confused flour beetle, *Tenebrio confusum* (J.) (Coleoptera: Tenebrionidae) and the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) (Aliniazee & Lindgren 1969), phosphine to *T. confusum* and *S. granarius* (Kashi & Bond 1975) and propylene oxide to *T. castaneum* (Navarro et al. 2004). The usefulness of CO_2 in enhancing the toxicity of EDN against insects is currently unknown but could be the subject of future studies.

The respiratory rate of insects influences the uptake and toxicity of fumigants. The uptake of HCN by the larvae of the mealworm beetle, *Tenebrio molitor* L. (Coleoptera: Tenebrionidae) was reduced by 66 % when spiracles were sealed with bee wax (Bond & Monro 1967), suggesting that more of the fumigant entered the insects through the spiracles. After comparing the toxicity of 13 fumigants to the confused flour beetle, *T. confusum* (J.) (Coleoptera: Tenebrionidae), cadelle beetle, *Tenebroides mauritanicus* (L.) (Coleoptera: Trogossitidae) and *S. granarius* (Coleoptera: Ciurculionidae), Bond and Monro (1961) found that most fumigants were less toxic to *T. confusum*, which had the lowest rate of respiration compared to the higher-respiring *T. confusum* and *S. granarius*. Previous studies by Bond (1956) also showed that susceptibility of *T. mauritanicus* to MB was directly associated with the rate of respiration in the beetle. To determine the penetrability and measure the physiological effects of EDN on adult *A. ferus* and *H. ligniperda*, studies should consider blocking spiracles of the insects.

The toxicity of EDN increased with increasing temperature from 10 to 20 ℃ for 3 hr when different life stages of *A. ferus* were exposed to the fumigant (Najar-Rodriguez et al. 2015a). In the brown powder-post beetle, *Lyctus brunneus* (Stephens) (Coleoptera: Bostrychidae), larvae were more susceptible to HCN at higher temperatures from 20 to 25 ℃ (Parkin & Busvine 1937).

The differential mode of entry of gaseous or powdery insecticides into various insects has been attributed to differences in spiracle size. Chaudhari (2016) suggested that insects with large spiracles may be more susceptible to insecticidal effects. However, he did not test this assumption in his experiments. Thus, in providing explanations to the observed differences in tolerance between *H. ligniperda* and *A. ferus* adults to EDN, it would be important to compare the differences in spiracle size between the two species.

2.5.2 The insect cuticle as insecticide route of entry

Despite the widely-held opinion that fumigants and other insecticides enter insects via the respiratory system, there is some evidence suggesting that penetration through the cuticle is also possible. For example, about 44 % of HCN passed through the cuticles of *T. molitor* (Bond 1961a). A quantity of MB fumigant was accumulated in *T. mauritanicus* larvae, despite having blocked the spiracles (Galley 1965) but whether all or some of the spiracles were blocked was not mentioned in the study. Additionally, a large quantity of dichlorvos vapour entered the flying desert locusts, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae) through the cuticle (MacCuaig & Watts 1963). Dichlorvos and nicotine also entered insects through the cuticle (Galley 1967). Matsumura (1963) also reported that Malathion passed through the legs of *P. americana*, showing that the cuticle was the main point of entry for the insecticide. Gerolt (1969) indicated that contact insecticides spread over the cuticle and entered insect bodies via the tracheae. Therefore, assessment of the detailed structure and composition of the insect cuticle as a barrier to EDN penetration into target species would aid our understanding of the fumigant mode of entry.

2.5.2.1 Factors that affect the uptake of insecticides through insect cuticle

The polarity of insecticides has been regarded as an important factor for cuticular penetration.

Given that the insect cuticle has both hydrophobic and hydrophilic properties, the effectiveness of an insecticide to penetrate the entire cuticle depends on its ability to pass through the hydrophobic or hydrophilic barriers and reach the target site (Webb & Green 1945). The toxicity of EDN to target insects may largely depend on its ability to penetrate the insect cuticle to gain access to the site of action. Therefore, it is important to assess the roles of cuticle layers and their properties in the penetration process.

Cuticular composition (Fig. 2.9) plays an important role in the uptake of fumigants (Pepper & Hastings 1943). Richards and Cutkomp (1946) observed that chitin facilitated the uptake of dichlorodiphenyltrichloroethane (DDT) into insect bodies by selective concentration through adsorption. In a study on the role of epicuticular wax in the houseflies, *M. domestica,* Srivastava (1965) found that melting point of the insects' epicuticular wax was lowered when insect cuticles were dissolved in DDT (15 %). It was observed that DDT was able to penetrate faster through the insect cuticle as it was in a semi-solid condition, due to the lowering of the melting point of the epicuticular wax. Assessment of the role of cuticular wax and the regulation of its melting point may help in enhancing EDN penetration into target insects and improving fumigant efficacy. I did not cover this aspect on my thesis but future studies may consider this.

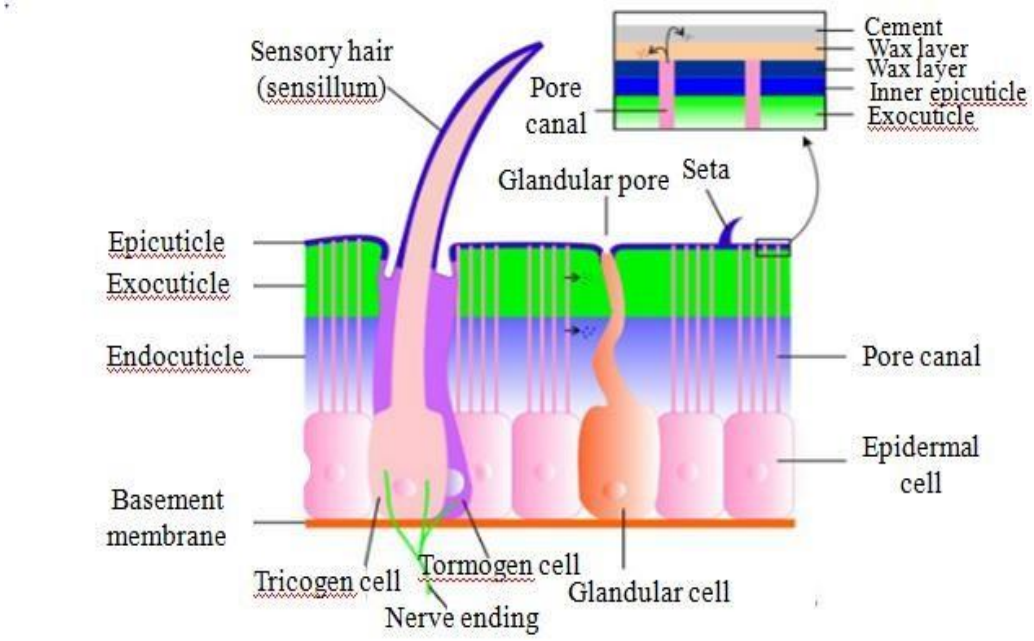

Figure 2.9 Section of an insect cuticle. Drawing by Jon Richfield, licensed under CC 3.0, re-labeled from the original.

Wilcoxon and Hartzell (1933) associated differences in insecticide susceptibility of insects with the thickness of their cuticles because thin layers such as sense organs, spiracles and intersegmental membranes allowed rapid penetration while thicker areas such as body segments and appendages were less permeable (Metcalf 1948). Clark and Triblehorn (2014) examined cuticle thicknesses of three cockroach species: *P. americana*, death's head cockroach, *Blaberus craniifer* Burmeister (Blattodea: Blaberidae) and *G. portentosa* and found that the latter possessed the thickest thoracic and abdominal cuticle, followed by *B. craniifer* and *P. americana*. Variations were also seen between exocuticles and endocuticles at the abdominal and thoracic regions within and between species, with both *B. craniifer* and *P. americana* having thicker thoracic exocuticle compared to the abdominal ones. In *G. portentosa*, the endocuticle layer possessed the largest percentage of the total thickness in both the abdominal and thoracic cuticle. Cuticle thickness influenced the susceptibility of insects to DDT, even though the chemical was believed to have penetrated more readily through the flexible cuticular membranes (Mukerjea 1953). Pyrethroid resistance in *Anopheles funestus* (Giles) (Diptera: Culicidae) was also associated with cuticle thickness (Wood et al. 2010).

The rate of insecticide uptake also varies according to insect species (Gratwick 1957) with delayed penetration in some species and excretion of chemicals in others (Alyokhin et al. 2008). Lin et al. (2012) explained that decreased penetration of insecticides through the cuticle may be attributed to modifications of the structure or composition of the cuticle because some insects may have additional cuticle layers. Lin et al. (2012) used semi-thin sectioning and transmission electron microscope (TEM) techniques to investigate structure and thickness of cuticle and fat bodies of susceptible and resistant strains of the oriental fruit fly, *B. dorsalis* larvae. Results showed that the cuticle of *B*-cypermethrin resistant strains was thicker than that of susceptible strains, and cuticular penetration of the insecticide into larvae of resistant strains was slower compared to that of susceptible strains. This research concluded that possession of additional cuticle layers in the resistant strains might have reduced the rate of uptake of insecticides into their body cavities. Therefore, comparative studies on the differences in cuticle thickness between adult *A. ferus* and *H. ligniperda* would be useful in understanding their contrasting tolerance levels to EDN.

2.6 The physiological and biochemical aspects of EDN toxicity

EDN is known to have the same mode of action as other cyanogen (e.g., HCN) and nitrile compounds (e.g., Acetonitrile (CH_3CN)) (Health 2002). The presence of a CN functional group in the chemical structure of EDN makes the CN^- anions toxic, when released. In human and other mammals, the fumigant dissolves rapidly within the lungs and passes directly into the circulatory system after inhalation (Ballantyne 1983). In the presence of body moisture, EDN breaks down into CN^- and cyanate (OCN^-) (Simeonova et al. 2004). The main route of metabolism in mammals is known to be the detoxification of rhodanase within the liver to form less toxic thiocyanate (Mousa & Davis 1991; Westley 1973).

EDN mode of action against insects is believed to involve hydrolysis to HCN, whereby cyanide ions (CN^-) bind to cytochrome c oxidase (COX) enzyme in the mitochondria of insect body cells (Hamel 2011), preventing the cells from having access to oxygen (Anseeuw et al. 2013) and causing "cellular asphyxiation" and death. However, recent studies conducted by Ramadan et al. (2020) on EDN mode of action against different life stages of the cigarette beetle, *Lasioderma serricorne* (F.) (Coleoptera: Ptinidae) and *R. dominica* suggest that COX may not be the main target for EDN toxicity. The results suggest that EDN mode of action might differ from species to species, and thus requires further investigations.

The symptoms of EDN toxicity to insects have not been described in the literature. However, HCN, a breakdown product of the fumigant is known to cause respiratory inhibition to a range of organisms. Bond (1961a) observed that rapid paralysis occurred in *S. granarius* after exposure, and HCN might have combined with COX. A slight respiratory inhibition was also reported in *T. molitor,* after the insects were fumigated with a lethal concentration. Similarly, cyanide caused 68-78 % respiratory inhibition in the codling moth, *Cydia pomonella* L. (Lepidoptera: Tortricide) (Pant 1958) and 75 % in *Gastrophilus* larvae (Levenbook 1951). The toxic effect of HCN on insects was attributed to the activation of a proteolytic enzyme like cathepsin (Bond 1961a).

Since HCN is the principal breakdown product of EDN with similar symptoms of inhibition,

exploration of symptoms exhibited by treated insects will be necessary for describing the toxicological effects of EDN on insects. Considering that EDN has shown various levels of toxicity across insect species, there is a need to conduct further studies on the quantification of its breakdown products within the final target insect organs. Considering that EDN may produce CO_2, HCN and formic acid, it is important to determine whether the toxicity of the fumigant is due to the combined effect of the break-down products or the individual break-down products.

2.7 Effect of EDN fumigation on insect behaviour

Insects can withstand insecticide exposure through various behavioural modifications such as avoiding or minimizing their encounter with the compounds (Jallow & Hoy 2005; Li et al. 2007). These behavioural adjustments may compromise insecticide efficacy (Lee et al. 2013). Therefore, the survival of insects to insecticides may greatly increase if their behaviour is altered to minimise or avoid exposure and uptake (Barson et al. 1992; Braga et al. 2011a). To determine the effectiveness of insecticides on insect pests, accurate assessment of behavioural effects on the overall efficacy is needed (Biondi et al. 2012). Although the effect of EDN on insect behaviour has not been explored, some studies have been conducted on the behavioural responses of insects to some other fumigants. To date, very few studies have investigated the significance of behavioural effects of lethal vs sub-lethal insecticide concentrations. Understanding the behavioural effects of EDN in this context can pose important implications in the implementation of fumigation programs using EDN for the control of target species.

Pimentel et al. (2012) studied the behavioural response of three populations of *R. dominica* to sub-lethal phosphine exposure and observed decreased walking activity of all three populations. Guedes et al. (2009b) also observed different behavioural responses between deltamethrin-susceptible and resistant strains of the maize weevil, *S. zeamais* and reported that walking behavioural patterns in response to the insecticide varied between the two strains. Behavioural analysis of the recovery trends of insects from the two different strains could explain the reasons behind the resistance and susceptibility status of both weevil strains. However, behavioural recording was not conducted as part of this investigation.

Behavioural recordings of the responses of insects to insecticide applications are now regarded as informative in evaluating the behavioural effects of insecticides, as they can reflect the

insecticide mode of action and the extent to which they influence insect behaviour (Haynes 1988). Pereira et al. (2014) assessed the walking response of three predatory bug species (*Orius tristicolor, Amphiareus constrictus* and *Blaptostethus pallescens* (Hemiptera: Heteroptera: Anthocoridae)) of the tomato leaf miner, *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), after exposure to eight insecticides, indicating that walking activity of these insects was reduced by the insecticides. In a study of the ground beetle, *Scarites quadriceps* Chaudior (Coleoptera: Carabidae), Prasifka et al. (2008) reported that exposure to lambda-cyhalothrin or tefluthrin increased total distance moved, maximum velocity and percentage of moving time. Repeated measurements on individual beetles indicated that their movement decreased after initial lambda-cyhalothrin exposure but increased following a second exposure. The parasitic wasp, *Encyrtus saliens* Prinsloo & Annecke (Hymenoptera: Encyrtidae) also increased its walking speed after contacting malathion residues (Hoy & Dahlsten 1984). Watson and Barson (1996) conducted a laboratory assessment of the avoidance behaviour of insecticide-resistant and susceptible strains of *Oryzaephilus surinamensis* (L.) (Coleoptera: Silvanidae) to pirimiphos-methyl, etrimfos, permethrin and the *N, N*-diethyl-*m*-toluamide (DEET) insect repellent. They recorded knock-down at 1 hr intervals for the first 7 hr and 24th hr after confining adults singly in untreated or half-treated arenas and exposing them to pirimiphos-methyl and etrimfos (25 or 250 mg/m^2) and permethrin (25 or 100 mg/m^2). Their results show that insect survival was enhanced by behavioural avoidance.

2.8 Target site insensitivity to insecticides

One of the most common insecticide resistance mechanisms in insects is reduced sensitivity of the target site. Various factors can cause such insensitivity of target sites to insecticides. For example, altered acetylcholine esterase (AChE) enzyme was responsible for the resistance of *Anopheles stephensi* (L.) (Diptera: Culicidae) (Safi et al. 2017) and Colorado potato beetles, *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae) (Ioannidis et al. 1992; Stanković et al. 2004; Wierenga & Hollingworth 1993) to organophosphates and carbamates. Additionally, the acetylcholine esterase-altered strain had less substrate affinity for both the enzyme and azinphosmethyl oxon than the susceptible strain (Clark 1997). However, AChE-induced resistance may be selective to insecticides. In a study by Wierenga and Hollingworth (1993), resistant *L. decemlineata* strain was markedly insensitive to arylcarbamates while another strain that was also affected by AChE became very insensitive to organophosphates but

not arylcarmabates. On the contrary, AChE alteration caused an insecticide-resistant strain of *L. decemlineata* to be more sensitive to both AChE-inhibiting α-chaconine and tomatine in tomatoes compared to the susceptible strain (Wierenga & Hollingworth 1992).

Target site alterations are not limited to only enzymes. For example, mutation in the voltage-sensitive sodium channel (VSSC) gene (M815I, T917I and L920F) have been identified as the main factor that caused nerve insensitivity in permethrin-treated *L. decemlineata* beetles (Kim et al. 2005; Lee et al. 2010). In another study, a mutation in acetylcholine receptors was cited as the cause of resistance in the brown plant hopper, *Nilaparvata lugens* (Stål) (Hemiptera: Delphacidae) to imidacloprid (Liu et al. 2006), but no mutation was found in the α-subunits of the target site that caused resistance in the beetles. In a permethrin-resistant *L. decemlineata* strain, one base pair mutation (C to T) caused an amino acid change in an α-subunit of the sodium channel (Alyokhin et al. 2008). In an azinphosmethyl-resistant strain, two mutations in AChE (S291G and R30K) reduced sensitivity of *L. decemlineata* to both azinphosmethyl and carbofuran insecticides (Kaplanoglu et al. 2017; Kim & Clark 2002; Kim et al. 2006). In the carbofuran-resistant strain, resistance was not caused by the presence of two mutations (I392T and S291G), but only one (S291G) caused high resistance to carbofuran and medium resistance to azinphosmethyl (Kim et al. 2007).

DDT and pyrethroid knockdown resistance in insects have been also associated with the substitution of amino acids in the target site (VSSC) of the insecticides (Rinkevich et al. 2013, Toshio 1985). It was also reported that the substitution of seven amino acids: I1011M, I1011V, V1016G, V1016I, F1534C, D1763Y and S989P caused resistance in *Aedes aegypti* (L.) (Diptera: Culicidae) (Chang et al. 2009; García et al. 2009; Kawada et al. 2009; Rajatileka et al. 2008; Saavedra-Rodriguez et al. 2007; Srisawat et al. 2010; Yanola et al. 2010). Out of the seven amino acids identified, substitutions involving only V1016G, V1016I, and F1534C had a very strong correlation with pyrethroid resistance (Du et al. 2013; Harris et al. 2010; Saavedra-Rodriguez et al. 2007).

It is not known whether adult *H. ligniperda*, which have shown high tolerance to EDN (Najar-Rodriguez et al. unpublished data) uses target site insensitivity as a mechanism of resistance to the fumigant. It is also unknown whether the insect uses more than one mechanism to tolerate EDN exposure. Studies of target-site resistance and other resistance mechanisms will help our understanding of the mechanism (s) behind the toxicity of EDN to both *A. ferus* and *H.*

ligniperda adults. Given that EDN is regarded as a promising alternative to MB for the control of insect pests (Park et al. 2014; Pranamornkith et al. 2014d; Ren et al. 2011), including adult *A. ferus* and *H. ligniperda* that have shown contrasting levels of tolerance (Najar-Rodriguez et al. 2015a; Pranamornkith et al. 2014c; Najar-Rodriguez et al. unpublished data), the reviewed literature presented above was taken as the basis for the research undertaken here, which dwells on the mechanisms behind this fumigant's mode of action against insects, using both *A. ferus* and *H. ligniperda* as model species.

CHAPTER 3 Toxicity of EDN to *Arhopalus ferus* and *Hylurgus ligniperda* in relation to Cuticle and Spiracle Features

3.1 Introduction

The efficiency of an insecticide depends on its ability to enter an insect though any possible means and to be able to reach the target site successfully (Richards & Cutkomp 1946). The most reported modes of entry of insecticides into insects are penetration through the cuticle, spiracles and oral intake (Sumita et al. 2016). Different types of insecticides utilize different routes of entry into their target insects, according to their chemical composition and properties. For example, contact insecticides first enter insect bodies through the cuticle and then proceed to the CNS using the haemolymph as a carrier (Grissom Jr et al. 1989; Matsumura 1963; Yu 2008). Petroleum oils were shown to penetrate the insect cuticle and accumulate in lipid-containing tissues before entering the nerve cells (Najar-Rodriguez et al. 2008). Insect spiracles are also the primary route of entry for various types of insecticides (Gerolt 1965; Gerolt 1969; Huber 1974; Sugiura et al. 2008). For instance, nicotine, dichlorvos, HCN and MB can penetrate the tracheal system and their efficacy can be enhanced by the presence of CO_2 concentrations (Bond 1961a; Jones 1938). Gerolt (1969) indicated that after application, carbon-14-dieldrin had spread over the cuticle of *M. domestica* and entered the insect body through the tracheal system. Malathion (Matsumura 1963) and parathion (Ball & Beck 1951) passed through both the cuticle and spiracles. However, the main route of entry for EDN into insects and the involvement of potential breakdown products, such as HCN, are not known.

3.1.1 Insect cuticle and insecticide resistance

The cuticle (Gorb 2001; Hepburn & Joffe 1976) serves as a barrier between the insects and toxic substances like fumigants and other insecticides (Gullan & Cranston 2014; Yu 2008). The morphological and biological properties of the insect cuticle have been extensively documented since the early 1900s (Casas & Simpson 2010). The detailed basic structures and physiology were first provided by Wigglesworth (1933) using the bloodsucking bug, *Rhodnius prolixus* Stål (Triatomidae, Hemiptera). However, cuticle structure and composition differ across species (Teal et al. 1999) and life stages within species (Aminlari et al. 1997). Generally, the cuticle is multi-

layered (Aminlari et al. 1997; Locke 2001) and comprises a basement membrane that separates the exoskeleton from internal organs of the insect body (Gullan & Cranston 2005). The major components of the envelope (outer layer) are waxes, lipids and sometimes carbohydrates. In some adult Coleoptera, the cuticle has a mesocuticle layer between the endocuticle and exocuticle (Cheng et al. 2009; Noble-Nesbitt 1991; Noh et al. 2016). The epicuticle contains lipids, lipoproteins and proteins and is therefore lipophilic; its surface is coated with lipids and wax. The endocuticle (often the thickest layer of the cuticle) (Locke 1974) comprises microfibers of chitin and protein for cuticle elasticity. Unlike the exocuticle, sclerotization does not take place in the endocuticle, which remains flexible (Gullan & Cranston 2005).

The pore canals stretch from the endocuticle to the periphery of the procuticle and transport mainly hydrocarbons of the integument to the outside of the body, forming the wax layer (Gullan & Cranston 2005) which maintains moisture inside the insect body (Blomquist & Bagnères 2010). It is lipophilic in nature and allows the penetration of non-polar insecticides (Locke 2001). The procuticle, mainly composed of chitin and cuticular proteins, is typically 10 μm to 0.5 mm thick (Cohen 2010; Gullan & Cranston 2005). Chitin is an amino-sugar polysaccharide, mainly containing β-(1-4)-connected units of N-acetyl-D-glucosamine (Cohen 2010). Differences in insecticide susceptibility across insect species are associated with the extent of cuticle sclerotization (Wigglesworth 1933), with less sclerotized layers allowing rapid penetration and highly-sclerotized ones being less permeable (Metcalf 1948).

Cuticle composition (Pepper & Hastings 1943) and thickness (Clark & Triblehorn 2014; Hartzell & Wilcoxon 1932; Wood et al. 2010) play important roles in the uptake of insecticides (Ahmad et al. 2006; Lin et al. 2012). Cuticle thickness influenced the permeability of DDT into the tomato moth, *Diataraxia oleracea* (L.) (Lepidoptera: Noctuidae), *T. molitor* and *P. americana* (Mukerjea 1953). A thick cuticle may reduce the penetration of insecticides but histological studies comparing cuticles across species in relation to fumigant tolerance have rarely been conducted. Delayed penetration of insecticides through insect cuticles has been explained on the basis of the possession of additional cuticle layers. For example, Lin et al. (2012) reported that delayed penetration of *B*-cypermethrin through the cuticle of resistant *B. dorsalis* larvae was due

to the presence of additional cuticle layers, as the cuticle of β -cypermethrin resistant strains was thicker than that of susceptible strains.

3.1.2 Insect respiratory system and insecticide efficacy

The insect respiratory system comprises a network of air-filled tracheal vessels that extend from the outer surface of the insects to their internal tissues (Wigglesworth 2012). The tracheoles are extended branches of tracheae, made of thin cuticle which is directly connected to the insect tissues and cells. Most insects take in gaseous substances through the tracheal system that connects to the outside environment through the spiracles, which may be actively opened or closed in response to abiotic factors (Chapman & Chapman 1998). Spiracles are openings located on the lateral sides of thorax and abdomen of insects (Contreras & Bradley 2010; Sparks Jr & Liu 2001), but the specific number and distribution may vary across insect species (Keilin 1944). In primitive coleopterans, spiracles appear in a row along both sides of the body and are usually 10 pairs: 1 mesothoracic, 1 metathoracic and 8 abdominal (Packard Jr 1874). However, a reduction in the abdominal spiracle number from 8 to 7 has been reported in adult bess bug, *Passalidae Lucanidae* (Leach) (Coleoptera: Lucanidae) and scarab beetles (Coleoptera: Scarabaeidae) (Ritcher 1969). Each spiracle is protected by bristles or setae (hairs) that prevent the entry of dirt (Richards & Davies 1997).

Many attempts have been made to classify spiracles based on various characteristics. The general classification based on the shape and type of the spiracles' closing apparatus was given by Tonapi (1958a). He also based another classification on muscle shapes such as the acicular occlusor of the first thoracic spiracles or parallel fibres in the cylindrical muscles of the abdominal spiracles. Spiracular structures in different insects vary in their regulatory spiracle-opening apparatus (Nikam & Khole 1989; Snodgrass 2018; Weber 1933). Spiracular openings of insects may also vary across species and between life stages. Reduction in spiracle openings might cause lower gas exchange through decreased respiration in insects (Contreras & Bradley 2010). Since the origin of the entire spiracle structure and the connected parts is the cuticle, the development of the closing apparatus primarily depends on the relative degree of sclerotization of the cuticle around the spiracle (Tonapi 1958a). The mechanism of closing and opening of spiracles is controlled by valves that are regulated by spiracular muscles (Mellanby 1934; Miller

1962; Ramsay 1935). Spiracular valves are regulated by loops that open spiracles when internal CO_2 pressure is high and O_2 pressure is a low, but close in response to high O_2 pressure (Förster & Hetz 2010; Heinrich 2015).

During respiration, gases including fumigants are transported within the tracheal system of insects through passive diffusion (Weis-Fogh 1964), but gaseous exchange through active ventilation is particularly common in beetles (Duncan & Byrne 2002; Duncan et al. 2010). The body size of insects influences their demand for oxygen requirements. For example, small insects possess a larger surface-area-to-volume ratio (Kühsel et al. 2017) and may have less demand for respiratory oxygen. In terms of tracheal investment and body size, larger insects are known to have lower mass-specific metabolic and gas exchange rates (Chown et al. 2007a).

In this chapter, I aimed to understand cuticle and spiracle features of *A. ferus* and *H. ligniperda* adults and how such differences may influence the toxicity of EDN. Information gathered will provide clues to the species-specific differences in the tolerance of these beetles to EDN. It will also add some basic information to the role of spiracle morphology in adaptations to xenobiotic conditions.

3.2 Materials and methods

Insects

Collection and storage of adult *A. ferus* were done according to Najar-Rodriguez et al. (2015a) with some modifications. Briefly, field-collected, non-sexed adults of mixed ages were hand-collected at night from timber processing mills near Nelson, New Zealand (from 2017 to 2019). To avoid mortality due to aggressive behaviour, less than fifty insects were placed into each 1-L plastic container with the lid perforated to enable air exchange and moist paper towels to provide humidity during transport. The containers were kept cool until delivered to the laboratory. In the laboratory, insects were kept at 10 ± 2 °C in the same plastic containers until experiments. Only live, active and undamaged insects were used in the experiments. One- to two-months-old adults of *H. ligniperda* were supplied by the insect breeding team at the Mount Albert branch of Plant and Food Research in Auckland. The insect colonies were laboratory-reared according to Clare

and George (2016a) and kept on artificial bark diet (Rogers et al. 2002) at 10 °C until experiments. To avoid overcrowding and in-fighting, about 20 insects were kept in each Petri dish (60 mm x 15 mm) with fresh diet.

Before fumigation experiments, adult *A. ferus* were individually kept in plastic vented queen bee cages (8 cm × 3 cm × 1 cm; supplied by Waireka Honey, Palmerston North) (Figure 3.1) and adult *H. ligniperda* were maintained in their original dishes. Insects from both species were kept in their containers at either 10 °C or 20 °C for 72 hr to get acclimatized to their respective fumigation temperatures.

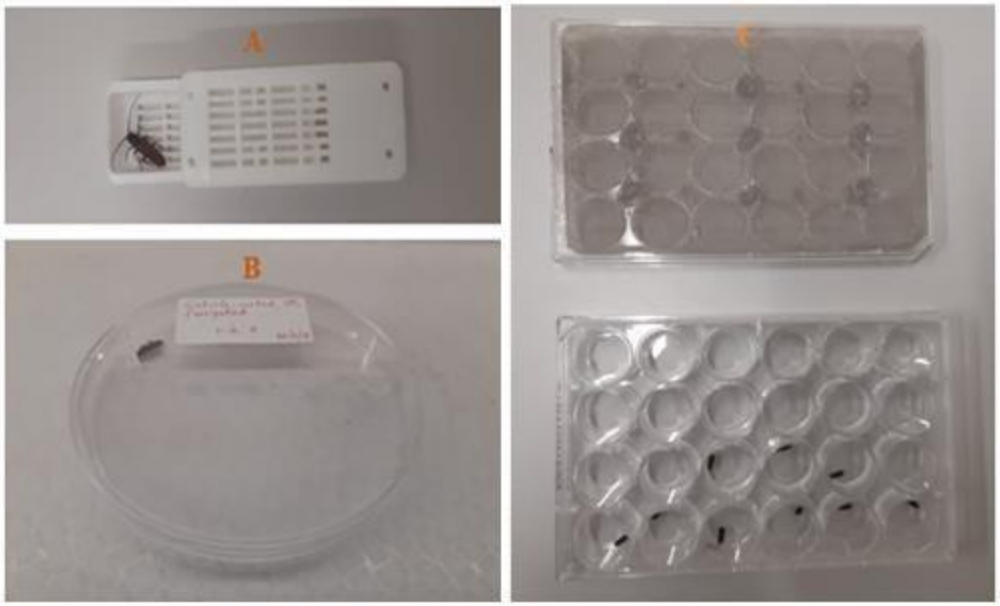

Figure 3.1 Containers for experiments. (A) Queen bee cage used to hold adult *Arhopalus ferus* for fumigation with ethanedinitrile; (B) Petri dish used to keep adult *Arhopalus ferus* insects during mortality counting, and (C) a 24 - well tissue culture plate and meshed lid containing 10 individuals of adult *H. ligniperda* used for experiments.

3.2.1 Measurement of total body surface area

The total body surface area of both beetle species was measured according to Simanton (1933), with some modifications. Briefly, 2 hr before dissection, the experimental insects were frozen at -20 °C overnight and removed from the freezer to thaw and relax the tissues. Each insect was weighed and then pinned in a waxed aluminium dissection tray (28 cm x 19 cm x 4 cm) filled with water (to avoid tissue dehydration). Then, elytra, wings, head plus the pronotum, legs, the rest of the thorax plus abdomen and antennae were all separated. To remove the internal organs, the posterior end of the abdomen was first punctured with a stainless-steel pin (R.M. Components P Ltd, India) and the digestive tract and other organs removed. Apart from the elytra and wings, each of the separated parts was pressed flat using two glass microscope slides (Biolab Scientific, New Zealand & Australia). To avoid damaging the spiracles, care was taken not to press the lateral body parts of the ventral thorax, too hard. Then, the area of each of the body parts was determined using a calibrated light microscope (LM) (Nikon SMZ 225, Japan). Twenty insects of each species were used for these measurements.

3.2.2 Determination of spiracle number, distribution and area

Insect spiracles were further dissected using a pair of scissors, forceps (BioQuip, CA 90220 USA) and the LM. The first thoracic (mesothoracic) spiracles were exposed for dissection by turning the insects such that the ventral side faced up (Fig. 3.4 A, B). For the second thoracic (metathoracic) spiracles, insects were positioned such that their lateral body sides were aligned dorso-ventrally. The abdominal spiracles were dissected by cutting the two lateral portions of the abdomen (where the spiracles were located). Dissected samples were mounted on a slide with a few drops of Lead (II) sulfide (PbS), mixed with glycerol (Najar-Rodriguez et al. 2008). Before mounting, the unwanted tissues such as fat bodies and tracheae were removed, and each spiracle was separated with fine needles and kept in a separate cavity block. The slides were kept dry at 40 °C and the spiracle areas measured using the LM and SmartSEM computer software program (Carl Zeiss AG, Oberkochen, Germany). Since the same insects were used for measuring the total body surface area and spiracle area, the cavity blocks of each insect's spiracles were given the same labels. To observe external movable structures (e.g., peritreme and setae), a different set of freshly-frozen beetles were observed under the LM. The number and distribution of spiracles were also recorded for each insect.

3.2.3 Insect cuticle sample preparation

The cuticle histological sample preparation for light microscopy was completed in accordance with Hayat (1981). To measure cuticle thicknesses, the cuticle of the same twenty adult insects from each species that were used in the spiracle and body surface measurements were examined. The middle metathoracic and fourth abdominal segments were isolated and prepared for histological work. The cuticle samples were fixed in a modified Karnovsky's fixative (3% gluteraldehyde (v/v) 2% formaldehyde (w/v) in phosphate buffer (0.1M, pH 7.2) for 2 hr, and washed thrice for 10 min each in 0.1 M phosphate buffer (pH 7.2) and put into glass vials (36 ml). Vials were stored at 4 ºC for 48 hr to allow the fixative to penetrate the tissues. Samples were post-fixed and stabilized in 1 % osmium tetroxide in 0.1 M phosphate buffer for 1 hr and buffer-washed three times for 10 min each, to remove the osmium tetraoxide. Cuticles were then dehydrated through graded acetone series (25, 50, 75, 95 and 100 %), for 10-15 min each, followed by two exchanges of 100 % acetone for 1 hr each before transferring them into a 50:50 resin: acetone solution and were stirred overnight. The following morning, fresh 100 % resin (Procure 812, ProSciTech Australia) was used to replace the solution for 8 hr, prior to embedding the samples in moulds with fresh resin and drying in a 60 °C oven for 48 hr.

Light microscope sections of cuticles were cut at 1 micron using an ultramicrotome (Leica EM UC7, Germany) and heat-fixed onto glass microscope slides. These were then stained with 0.05 % toluidine blue for approximately 12 s to facilitate the identification of cuticle layers. The cuticle samples were then mounted perpendicularly to the mounting stub and placed in position using a pair of forceps.

3.2.3.1 Measurement of cuticle thicknesses

Total cuticle thickness measurements, as well as the thickness of the epicuticle, exocuticle and endocuticle layers of the prepared samples, were performed using a Qcolor3 Olympus digital camera on a light microscope (Olympus SZX-ILLD2-200, Olympus Corporation, Tokyo, Japan). Fine sections of the middle metathoracic and fourth abdominal segments (both dorsal and ventral) were selected for measurements and comparison. The points were specifically chosen because of the uneven nature of the cuticle thickness even on the same body segment. Cuticle thickness was measured from the most exterior layer to the last layer, using the two-point

distance calculator/converter tool from the SmartSEM computer software program (Carl Zeiss AG, Oberkochen, Germany). Each point was measured three times at the highest magnification, which produced the best view on the computer screen. The three measurements were then used to calculate an average measurement of cuticle thickness at each point. A total of 4 points, one each from the middle, dorsal and ventral fourth abdominal and metathoracic segments were measured per sample. A total of 240 cuticle measurements were taken for each species.

3.2.4 Spiracle blocking

To quantify the effect of spiracle penetration on the toxicity of EDN to our target insects, each adult (except the unblocked controls) was first anaesthetised on a frozen wax tray (27 cm × 17 cm × 1.5 cm; previously kept at -20 °C overnight) so that the spiracles could be blocked with nail polish (Manicare®, France). Ten individuals from each species were allocated to each of the following treatments: 1) all thoracic spiracles blocked and fumigated with EDN; 2) all thoracic spiracles blocked but not fumigated, 3) all abdominal spiracles blocked and fumigated, 4) all abdominal spiracles blocked but not fumigated, 5) all spiracles blocked and fumigated, 6) all spiracles blocked but not fumigated, 7) all spiracles unblocked, but fumigated, and 8) all spiracles unblocked and not fumigated. To expose the abdominal spiracles, the insects' elytra and hind wings were raised open using a stainless-steel pin with the aid of a dissecting microscope (Leica Microsystems Ltd, Germany). The thoracic spiracles were exposed by placing insects laterally onto the tray. After that, the nail polish was applied to block the spiracles holes (Fig. 3.2). The nail polish was then allowed to dry for 5 min at room temperature before elytra and wings were left to fold back as per normal. After blocking, *A. ferus* adults were placed individually in their plastic vented queen bee cages while *H. ligniperda* were kept in 24-well tissue culture plates (Interlab, Johnsonville, Wellington), one insect per well, without diet. The time at which the first and last spiracle on each insect was blocked was recorded.

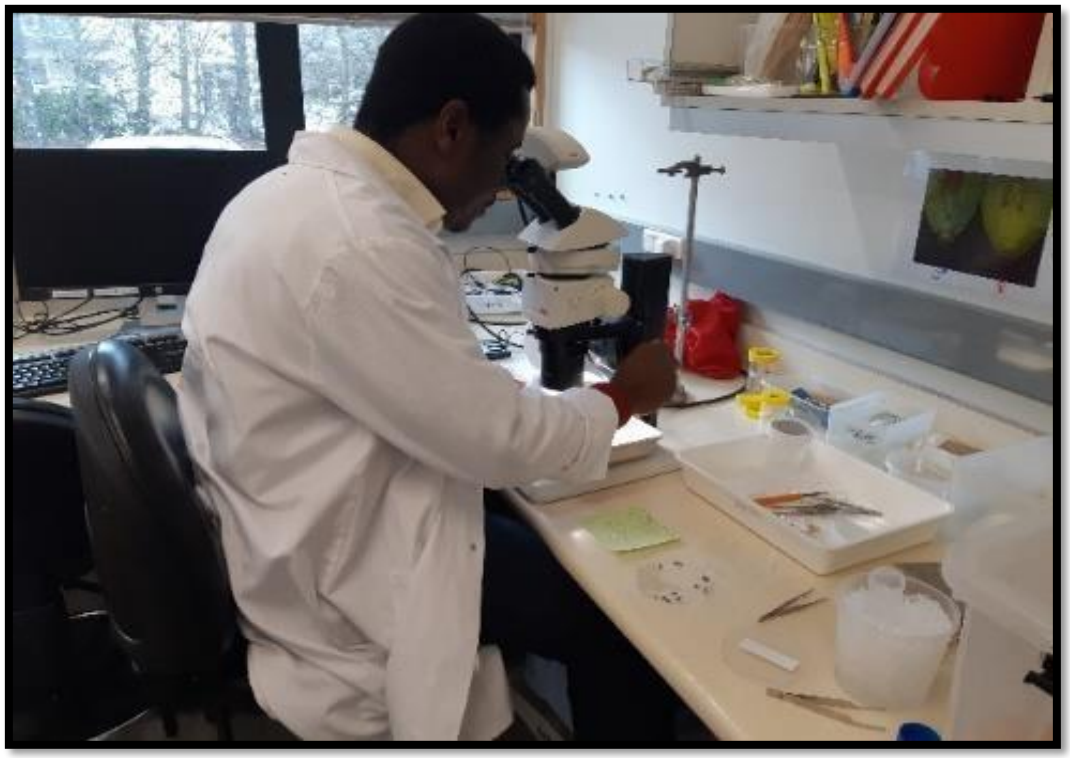

Figure 3.2 Insect spiracle-blocking with the aid of a dissecting microscope.

3.2.5 Cuticle surface coating

To measure the effect of cuticle penetration on the toxicity of EDN, each adult (except the unblocked controls) was anaesthetised as previously mentioned. The entire insect cuticle (except the spiracles and eyes) was coated by applying five layers of the same nail polish described above, using a fine brush under a dissecting microscope. After coating each insect, the nail polish was allowed to dry for 5 min. The time at which each insect's cuticle was coated was recorded. After the application of nail polish, adult *A. ferus* were placed individually in their plastic vented queen bee cages, while *H. ligniperda* were transferred to 24-well culture plates, with one insect per well, and without diet. Once coated, groups of 10 insects each were allocated at random to one of four treatment groups as follows: 1) cuticle coated and fumigated with EDN, 2) cuticle coated but not fumigated, 3) cuticle uncoated and fumigated with EDN, and 4) cuticle uncoated and not fumigated.

3.2.6 Fumigations with EDN

Fumigations were carried out in 28-L chambers (Labconco® desiccators, Kansas City, Missouri, USA). To get the most average-based responses of EDN that could kill 50 % of the test insects, the lethal concentrations (LC_{50}) were calculated, tested and confirmed. For *A. ferus*, LC_{50} of 2.8 g/m^3 at 20 °C and 3.2 g/m^3 at 10 °C, for 3 hr, were used based on results reported by Najar-Rodriguez et al. (2015a). For *H. ligniperda*, LC_{50} used were 120 g/m^3 at 20 °C and 150 g/m^3 at 10°C, for 3 hr, according to Najar-Rodriguez et al. (Unpublished data). Fumigations were conducted within 1 hr of spiracle-blocking or cuticle-coating (Fig. 3.3). To prevent desiccation of insects during the 3 hr fumigations, a wet sponge (5 cm × 5 cm × 1 cm) was placed at the bottom of each chamber. Treatment groups that were not fumigated, both spiracle-blocking and cuticle-coating experiments, were placed in separate fumigation chambers without the fumigant. Each experiment was replicated three times at either 10 °C or 20 °C. Each replicate was also conducted across different weeks to account for temporal variation in insect responses to EDN.

Figure 3.3 Fumigations of insects with ethanedinitrile in 28-Litre chambers.

During fumigation, concentrations of EDN in the chambers were monitored by collecting gas samples at 0, 1, 2 and 3hr from the treated space. Concentrations of EDN were quantified using an EDN-validated method Brierley et al. (2019). Briefly, quantification was done using an Agilent 7890A GC fitted with a FID and a capillary column (30 m x 0.53 mm) GS-Q (Agilent Technologies Inc., Auckland, New Zealand). The oven and front detector temperatures were 150 °C and 300 °C, respectively. Fumigant samples were withdrawn from the chambers using a gas-tight syringe (Valco Instruments Co., Texas, USA). Though 5 ml samples were injected, only 1 ml sub-sample was analysed. During fumigation each day, five-point calibration curves comprising dilutions of pure EDN with air were established. After fumigation, chambers containing the insects were aerated for 30 min, with the aid of a vacuum pump connected to an EDN-scrubbing system (Nordiko Quarantine Systems Pty Ltd, Sydney) to ensure no fumigant remained in the chambers. Insects were then removed from the chambers and placed under the fume-hood (for up to 1 hr), before being transferred into clean and freshly-labelled Petri dishes (150-mm diameter; for *A. ferus*, Fig. 3.1 B) or 24-well tissue culture plates (for *H. ligniperda*, Fig. 3.1 C) for mortality assessments. Both Petri dishes and plates were kept at a 20 °C temperature-controlled room.

Insects were assessed as either dead (no movement observed) or alive (exhibited any type of movement in response to physical stimuli) at 2, 4, 8, 12, 24 and 48 hr post-fumigation for both *A. ferus* and *H. ligniperda* (Najar-Rodriguez et al. 2015a; Pranamornkith et al. 2014d; Pranamornkith et al. 2014e). To determine mobility more accurately, insects were gently disturbed by touching their antennae and limbs (using a fine brush) and closely observed for any type of movement. Any beetle that showed movement when disturbed in this way was considered to be alive.

3.2.7 Statistical analyses

The comparison of total surface area and spiracles area between the two species was analyzed using generalized linear model (GLM) with gamma distribution (link = log). Average individual spiracle areas were also compared using independent samples T-test. For the cuticle histology experiments, analyses of variance with species, body parts, and position as main factors plus their interactions were conducted using Genstat (version 19; Goedhart 2018). Post-hoc

comparisons on the difference between the total cuticle (i.e., the sum of individual cuticle thicknesses from dorsal and ventral positions) thicknesses of *A. ferus* vs *H. ligniperda* were analyzed using the least significant difference (LSD) test. The differences between the mean total cuticle thicknesses taken from dorsal and ventral positions of the abdomen and thorax of both species were determined by comparing mean differences (MD) with LSD values. If MD was greater than the LSD values, a significant difference between the mean total cuticle thicknesses between both species was declared.

The effects of spiracle-blocking or cuticle-coating were analysed using a binomial GLM, with blocked or coated, temperature, and fumigated/not fumigated as factors. The spiracle-blocking and cuticle-coating experiments were analysed separately. Effects for temperature, fumigated vs non-fumigated and blocked vs unblocked spiracles (coated vs uncoated cuticles) were fitted, along with their interactions. Multiple means were compared using pairwise likelihood ratio tests (LRPAIR Biometris GenStat Procedure Library Manual, 19th Edition (Goedhart & Thissen 2018)), at $p < 0.008$ ($p = 0.05$ Bonferroni corrected). Graphs were produced by the R package ggplot2 (Wickham 2016) and GLM models were fitted with GenStat 18th Edition (Payne et al. 2015).

3.3 Results

3.3.1 Spiracle number, location, area, and body surface area

Striking morphological and structural differences between *A. ferus* and *H. ligniperda* were found. Although both species possessed nine prominent spiracles (two thoracic and seven abdominal, Fig. 3.4 A, B), total spiracle size in *A. ferus* was significantly larger than that for *H. ligniperda* (t= -7.15, *df* = 39, *p* = 1.5e-08) (Fig. 3.5A). Similarly, adult *A. ferus* had significantly larger total body surface area (t= -26.47, *df* = 39, *p* < 2.0e-16) (Fig. 3.5B) and surface area-to-spiracle-size ratio (324.31/0.89) than adult *H. ligniperda* (32.79/0.12).

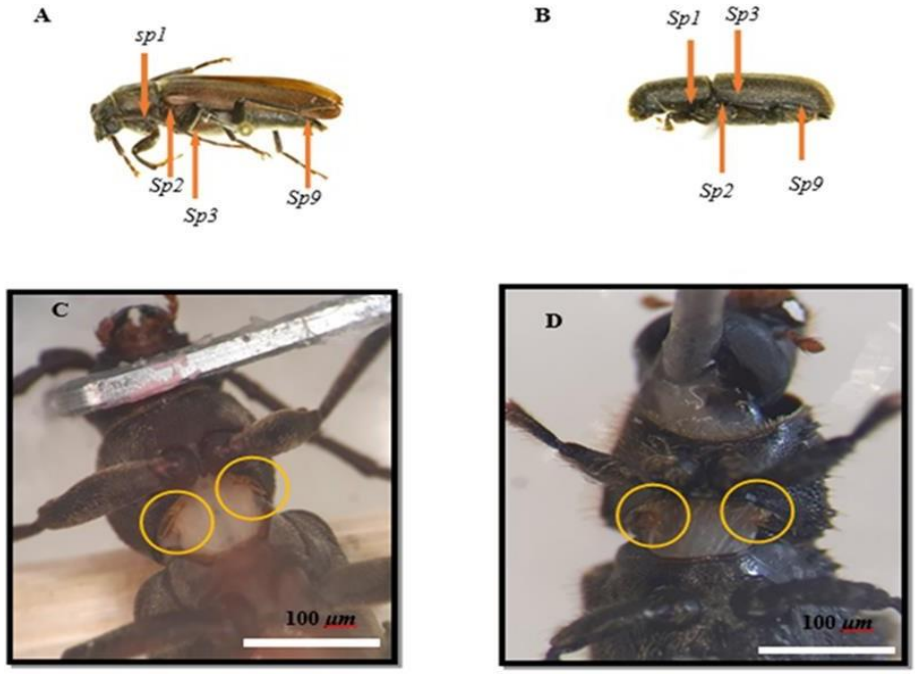

Figure 3.4 Light microscopy images showing the location of spiracle openings in adult *A. ferus* (A) and *H. ligniperda* (B): sp1, mesothoracic spiracle; sp2, metathoracic spiracle; sp3–9, abdominal spiracles. Intersegmental membranes between the prothorax and mesothorax, showing the ventro-laterally located mesothoracic spiracles of *A. ferus* (C) and *H. ligniperda* (D). Images were taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

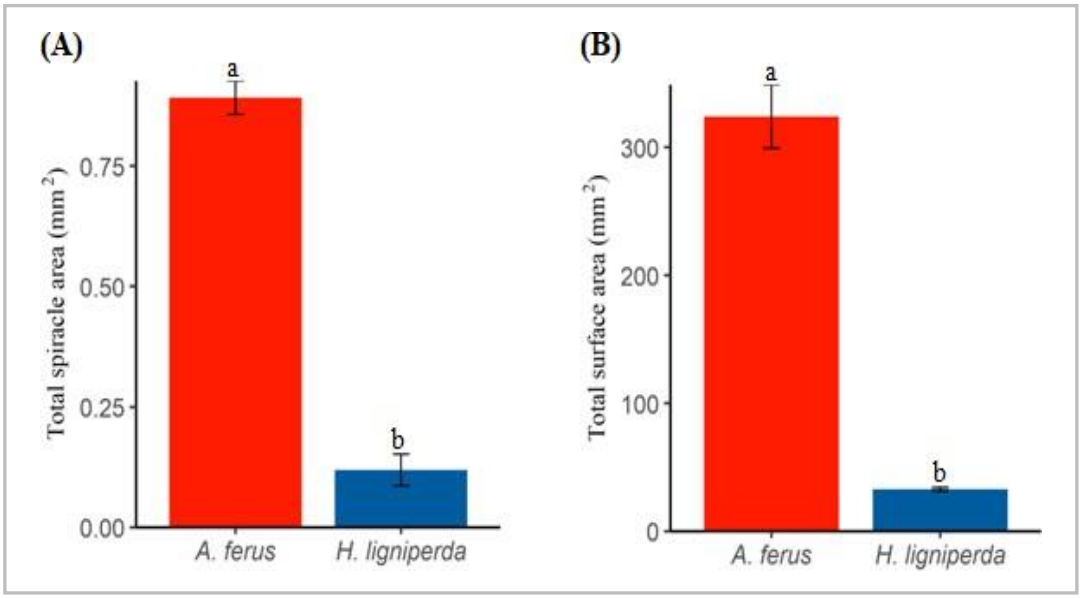

Figure 3.5 Comparison of (A) total spiracle area (mm^2) and (B) total body surface area of *A. ferus* and *H. ligniperda*. Different letters on bars in each category indicate significant differences at α = 0.05. Mean ± S. E. are shown (n = 20 individuals for each species).

3.3.2 Thoracic spiracles

The external and internal closing mechanisms of the different spiracles also varied between the two species. The first thoracic (mesothoracic) spiracles were the largest of all spiracles in *H. ligniperda* (Fig. 3.7). In *A. ferus*, they were the second largest, after the first abdominal spiracles (Fig. 3.7). These spiracles (usually concealed in most insects) were found in both species to be residing in the peritreme sclerite and situated ventro-laterally in the inter-segmental pleural membranes between the prothorax and mesothorax. Mesothoracic spiracles were significantly larger in *A. ferus* (0.1696 mm^2 ± 0.0042) than *H. ligniperda* (0.0674 mm^2 ± 0.0238) (t = 4.2, *df* = 38, *p* = 0.00067). Spiracular setae in *A. ferus* were bi-pectinate (i.e., feather-like and on both sides of the main shaft, Fig. 3.6 A) and more separated, compared to those of *H. ligniperda*, which were simple acuminate (ie., narrowed to a point) and more compact (Fig. 3.6 B). The setae were located on both the lower and upper spiracular lids of the mesothoracic spiracle of *A. ferus*, but only on the lower lid of *H. ligniperda*. The peripheral edge of the peritreme sclerite of *H. ligniperda* appeared more sclerotized than that of *A. ferus*. In both species, the spiracular

openings were situated transverse to the peritreme sclerite. Three separate tracheal openings appeared at the base of the atria and each atrium opened into a trachea.

The second thoracic (metathoracic) spiracles (Fig. 3.6 C, D), located lateral to the body and just above the middle legs of both species were enclosed in a well sclerotized cuticle. These spiracles were significantly larger in *A. ferus* (0.1199 mm^2 ± 0.0045), compared to *H. ligniperda* (0.03360 mm^2 ± 0.0089) ($t = 8.6$, $df = 38$, $p < 0.05$). The lids in both species had no setae. In *H. ligniperda*, the spiracles were more confined in a more sclerotized region. In both species, the atrial chambers extended just below the spiracular opening. Each atrium was also connected to a trachea.

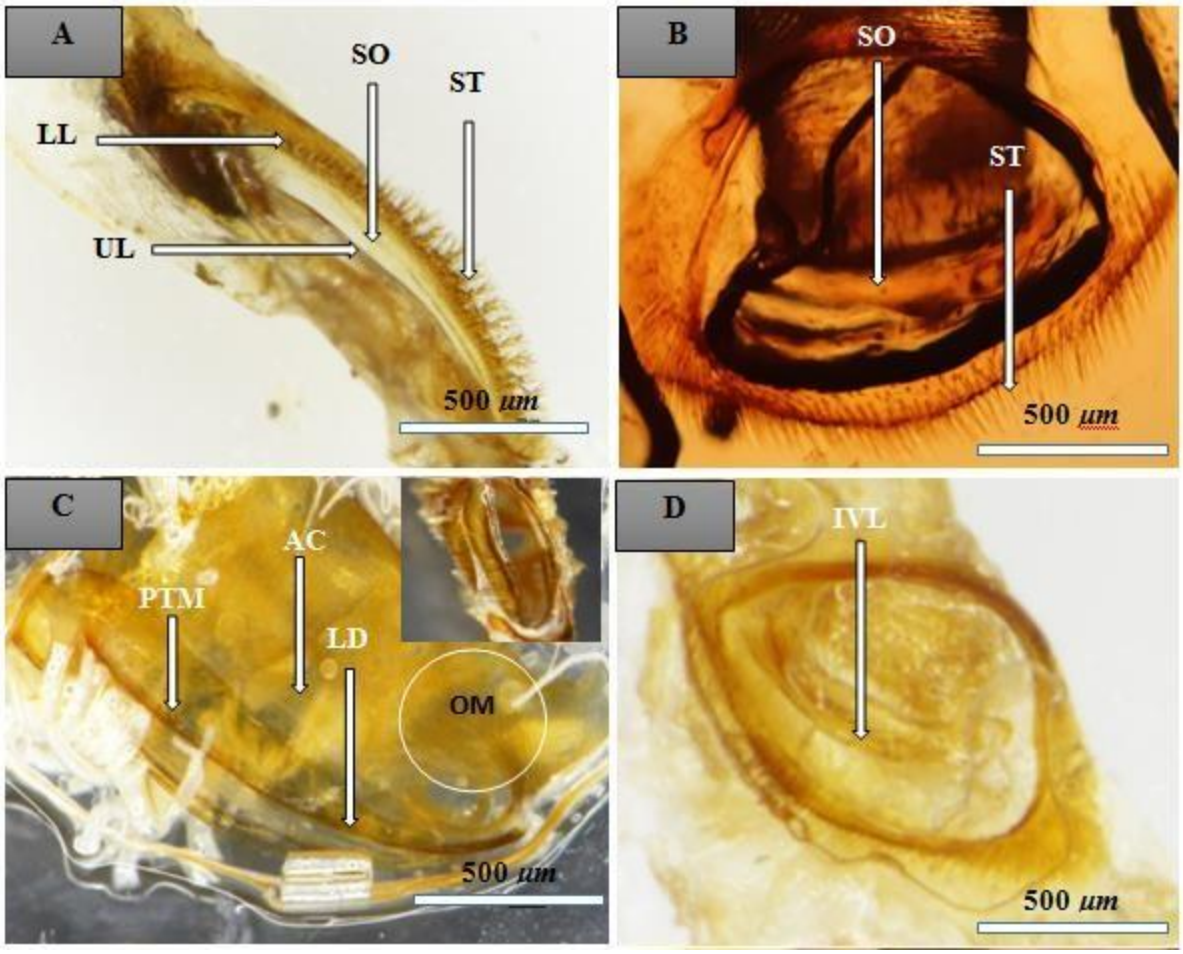

Figure 3.6 Light microscopy images showing the structure and closing mechanisms of the mesothoracic (A, B) and metathoracic spiracles (C, D) of adult *A. ferus* and *H. ligniperda*, respectively. AC, atrial chamber; IVL, inner valve; LD, lid; LL, lower lid; OM, oclusor muscle; PTM, peritreme; ST, setae; SO, spiracular opening and UL, upper lid. Images were taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

3.3.3 Abdominal spiracles

Each species has 7 abdominal spiracles which appeared dorsal to the hind legs and were located on the dorso-lateral sides. In *A. ferus*, the first pairs of these spiracles were elongated, appeared "slipper-shaped" and narrow (Fig. 3.8 A), with the opening being surrounded by a regulatory apparatus situated between the atria. In *H. ligniperda*, these spiracles were round in shape and had very small inner spines and valves (Fig. 3.8 B). In both species, the first pairs of abdominal spiracles were the largest. The first abdominal spiracles were significantly larger in *A. ferus*,

(0.4925 mm^2 ± 0.0111) than in *H. ligniperda* (mm^2) (0.0123 mm^2 ± 0.0008) (t = 43.1, *df* = 38, *p* < 0.05). Other abdominal spiracles were on the lateral sides of the terga of the abdominal segments and positioned under the elytra. The last abdominal spiracles in *A. ferus* were significantly larger (Fig. 3.7, 3.8 C) (0.0139 mm^2 ± 0.0038) than those in *H. ligniperda* (Fig. 3.7, 3.8 D) (0.0002 mm^2 ± 0.00005) (t = 3.6, *df* = 38, *p* = 0.00088), and all possessed spines (Fig. 3.8 C). The atria of all abdominal spiracles opened into the major atria, which were further connected to the trachea.

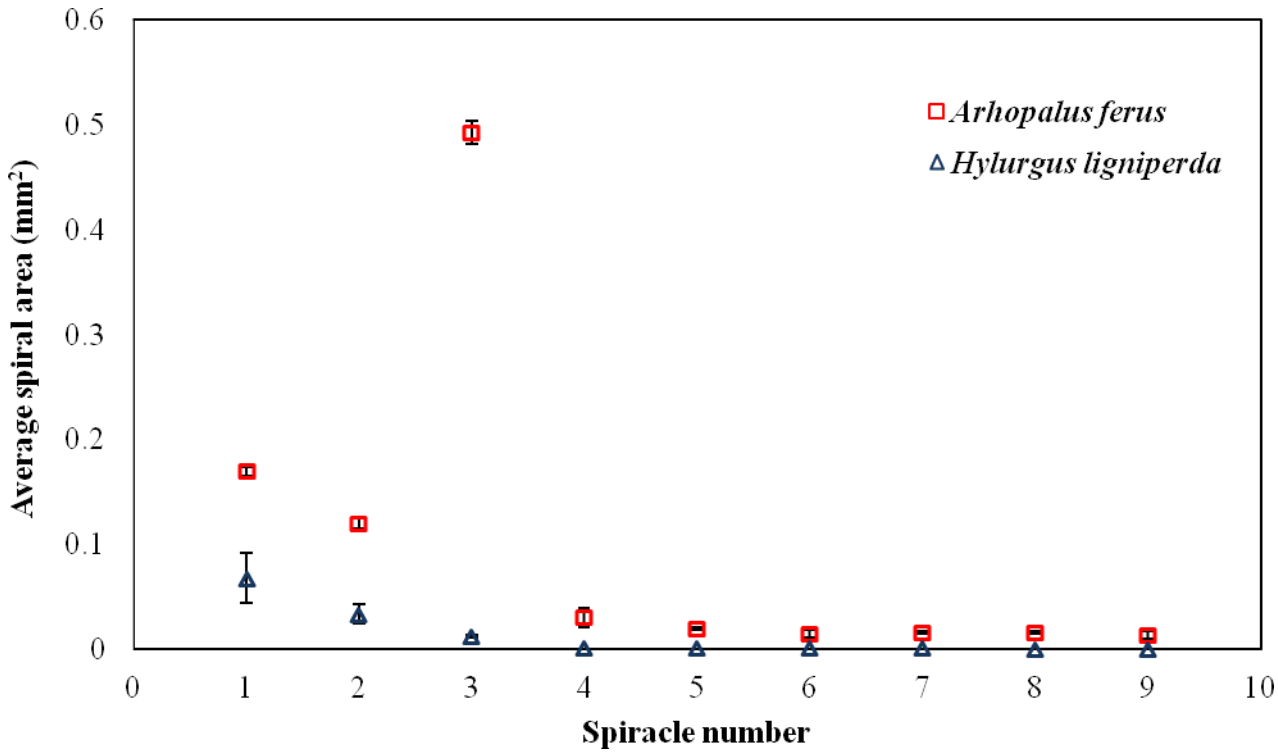

Figure 3.7 Comparison of area (mm^2) of spiracles 1-9 in *A. ferus* and *H. ligniperda* adults. Mean ± S. E. are shown (n = 20 individuals for each species).

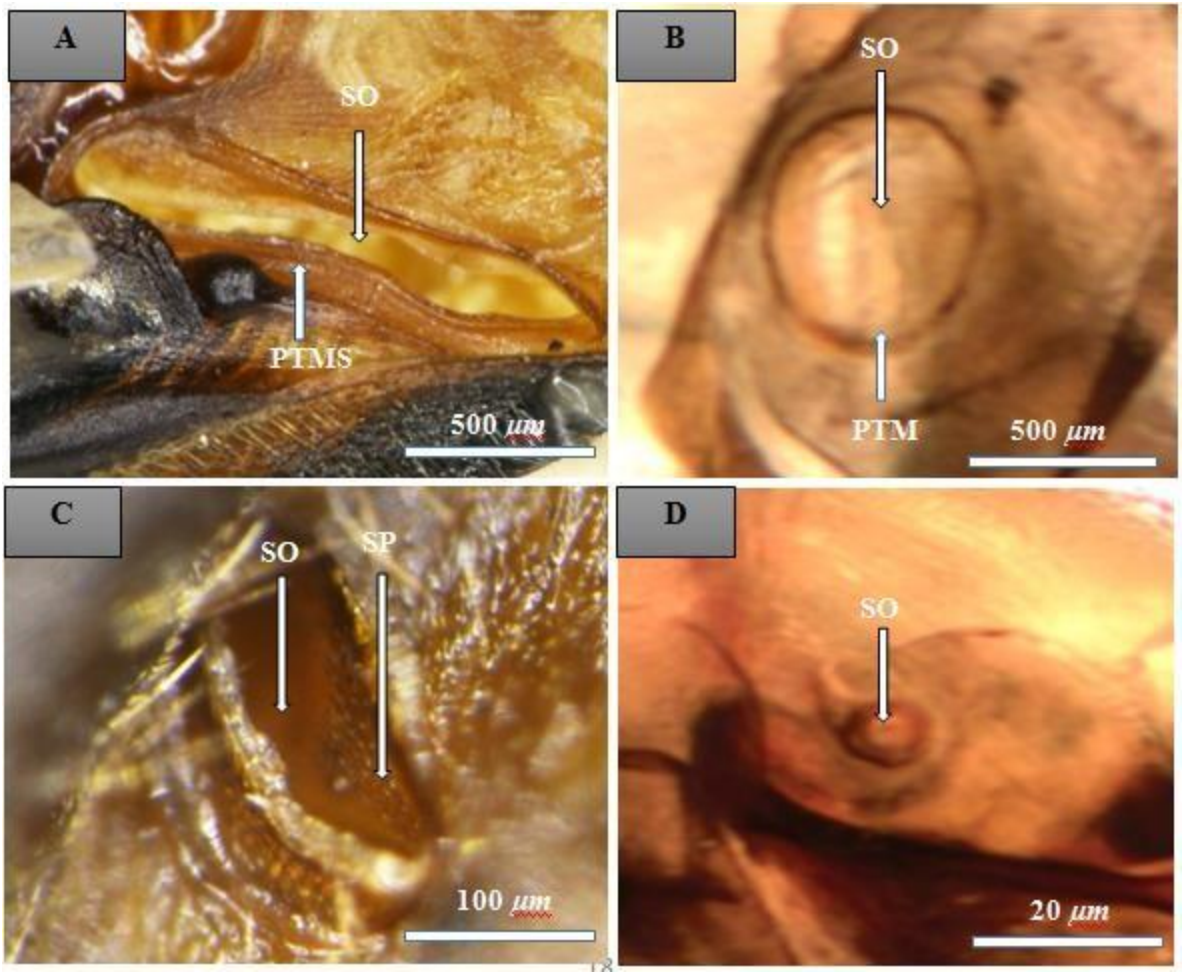

Figure 3.8 Light microscopy images showing the structure and closing mechanisms of the first abdominal (A, B) and last abdominal (C, D) spiracles of adult *A. ferus* and *H. ligniperda*, respectively. LD, lid; PTM, peritreme; PTMS, peritreme sclerite; SO, spiracular opening; ST, setae and SP, spine. Images were taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, NZ.

3.3.4 Cuticle thickness from body parts and positions

Of both species, *A. ferus* is significantly thicker at the dorsal abdomen (Fig. 3.9). Also, the ventral abdomen and ventral thorax of *H. ligniperda* were significantly thicker than those for *A. ferus*. The total cuticle thickness of the dorsal thorax of *A. ferus* was also significantly thicker. Also on the same insect species, total cuticle thicknesses from the dorsal and ventral parts of the mid-mesothorax and fourth abdominal body parts of both species were different (Fig. 3.10, Fig. 3.10 (B, C, E, and F). For example, in *A. ferus*, the dorsal thoracic cuticle was thicker than the

ventral thoracic one. Generally, the cuticle of *H. ligniperda* had more chitin layers, compared to that of *A. ferus* (Fig. 3.10 B, C, E, and F).

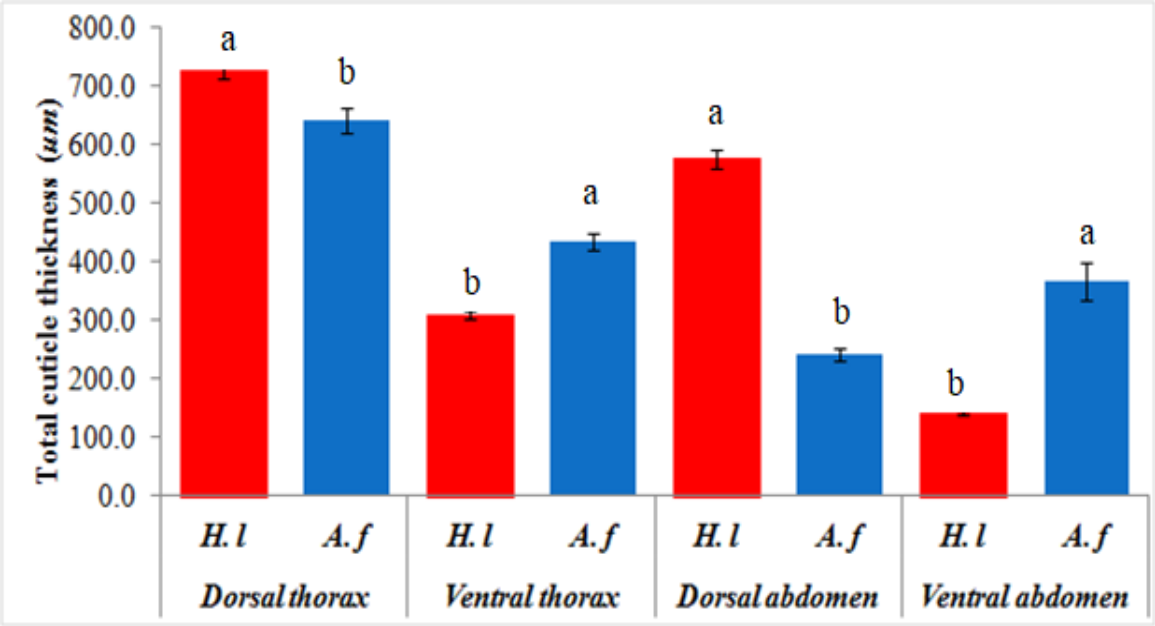

Figure 3.9 Comparison of adult *Arhopalus ferus* (*A. f*) and *Hylurgus ligniperda* (*H. l*) cuticle morphology. Measurements were taken from the mid-dorsal and mid-ventral positions of the thoracic and abdominal cuticles of 20 individuals from each species. Different letters on bars in each body part and position indicate significant differences at α = 0.05. Mean ± S. E. are also shown.

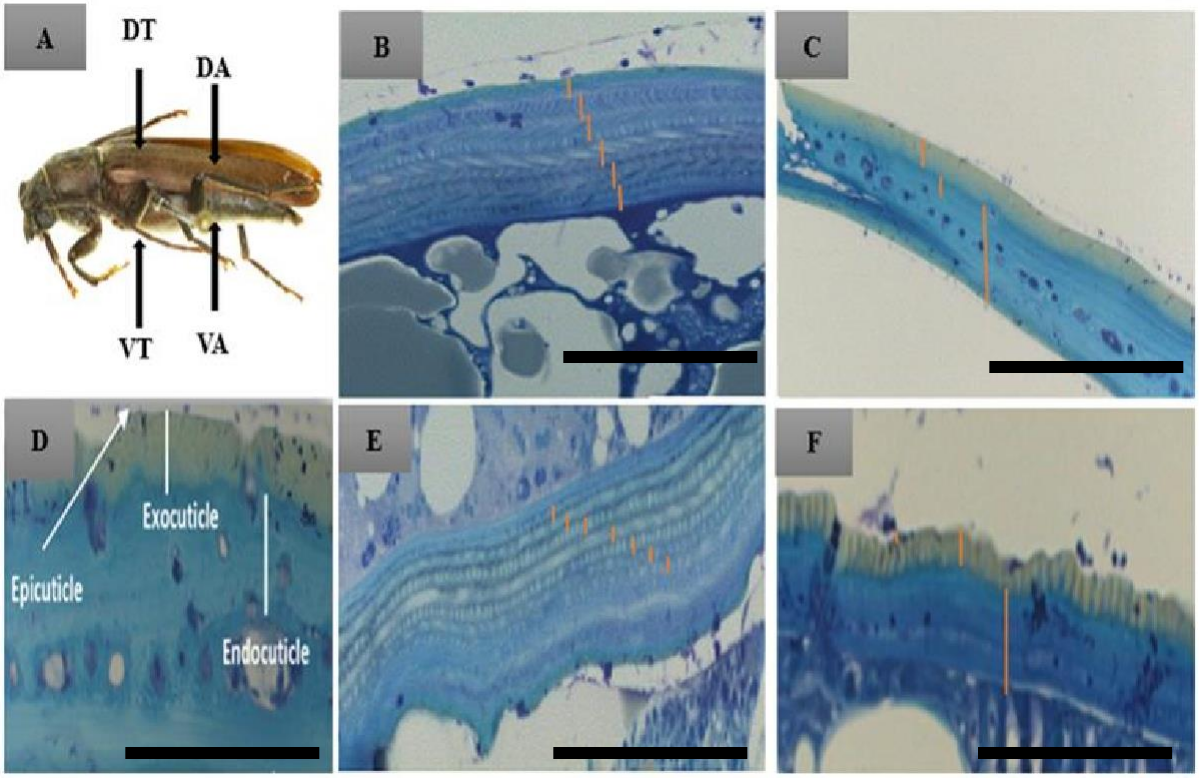

Figure 3.10 (A) Adult *A. ferus* showing regions where cuticle samples were taken from DT, dorsal thorax; DA, dorsal abdomen; VT, ventral thorax, and VA, ventral abdomen. (B) and (C) are transverse sections of toluidine blue-stained cuticle sections of the 4th dorsal thoracic segments of *H. ligniperda* (40x) and *A. ferus* (20x), respectively. (D) shows the epicuticle, exocuticle and endocuticle layers of a sample thoracic cuticle of *A. ferus*, (E) and (F) are sections of the abdominal cuticle of a *H. ligniperda* and *A. ferus*, respectively. Scale barsfor all sections are 100 *µm*. Orange bars indicate chitin layers seen in sections. Images were taken using a Leica SP5 DM6000B Scanning Confocal microscope, Massey University, Palmerston North, New Zealand.

3.3.5 Spiracle blocking

Because the effect of fumigation temperature (10 ºC and 20 ºC) on mortality was not significant at 48 hr for *A. ferus* (binomial GLM: deviance = 0.1, *df* = 1, *p* = 0. 765) and 96 hr for *Hylurgus ligniperda*) (binomial GLM: deviance = 0.0, *df* = 1, *p* = 1.000), data were pooled from both temperatures for analysis. However, blocking the spiracles of *A. ferus* (binomial GLM: deviance = 71, *df* = 3, $p < 0.001$) or fumigating the insects (binomial GLM: deviance = 262.5, *df* = 1, $p < 0.001$) significantly affected their mortality. Likewise, blocking the spiracles of *H. ligniperda* (binomial GLM: deviance = 39.6, *df* = 3, $p < 0.001$) or fumigating them (binomial GLM: deviance = 405.7, *df* = 1, $p < 0.001$) significantly affected their mortality. For both species, all interactions involving fumigation temperature, fumigation and spiracle blocking were not significant ($p > 0.001$).

The results in Table 3.1 show that mortality in all spiracles blocked and fumigated insects were significantly higher than those for the unblocked and fumigated controls, as all spiracle-blocked insects failed to recover at 48 hr (*A. ferus*) or 96 hr (*H. ligniperda*) after EDN fumigation. For *A. ferus*, the mortality for fumigated insects with all thoracic spiracles blocked was statistically lower than the unblocked and fumigated controls. For *H. ligniperda*, mortality for fumigated insects with all thoracic spiracles blocked was slightly higher than the unblocked and fumigated controls, but not statistically significant (Table 3.1). No significant effects were found between the mortality of fumigated *A. ferus* with all abdominal spiracles blocked (though higher) and their unblocked and fumigated controls (though lower). However, blocking all abdominal spiracles of *H. ligniperda* and fumigating resulted in statistically higher mortality, compared to the unblocked and fumigated controls. Though blocking all abdominal spiracles of *A. ferus* and fumigating resulted in higher mortality, compared to the unblocked and fumigated controls, this difference was statistically insignificant.

Table 3.1 Effect of spiracle-blocking on the mean mortality (%) of adult *Arhopalus ferus* (at 48 hr) and *Hylurgus ligniperda* (at 96 hr), after 3-hr exposure to ethanedinitrile (EDN) fumigations (pooled data from 10 and 20 ºC).

Treatment	% Mortality (± SE) of *A. ferus*	% Mortality (± SE) of *H. ligniperda*
All thoracic spiracles blocked, fumigated	18.33 ± 5.00 b	48.33 ± 6.45 c
All abdominal spiracles blocked, fumigated	68.33 ± 6.01 c	100.00 ± 0.01 d
All spiracles blocked, fumigated	100.00 ± 0.01 d	100.00 ± 0.01 d
All spiracles unblocked, fumigated	51.67 ± 6.45 c	46.67 ± 6. 44 c
All thoracic spiracles blocked, not fumigated	0.00 ± 0.01 a	0.00 ± 0.00 a
All abdominal spiracles blocked, not fumigated	0.00 ± 0.00 a	0.00 ± 0.00 a
All spiracles blocked, not fumigated	15.00 ± 4.61 b	5.00 ± 2.81 b
All spiracles unblocked, not fumigated	0.00 ± 0.00 a	0.00 ± 0.00 a

Treatments with the same letter within a column are not significantly different from each other ($p < 0.01$), LSD for 10 individuals per replicate.

3.3.6 Cuticle coating

There was no significant effect of fumigation temperature (10 ºC and 20 ºC) on mortality at 48 hr for *A. ferus* (binomial GLM: deviance = 1.3, $df = 1$, $p = 0.248$) and 96 hr for *Hylurgus ligniperda*) (binomial GLM: deviance = 0.0, $df = 1$, $p = 0.199$). Therefore, data were pooled from both temperatures for analysis. Cuticle-coating did not significantly affect the mortality of *A. ferus* (binomial GLM: deviance = 2.8, $df = 1$, $p = 0.094$) and *H. ligniperda* (binomial GLM: deviance = 9.0, $df = 1$, $p = 0.011$). However, fumigation significantly affected the mortality *A. ferus* (binomial GLM: deviance = 262.5, $df = 1$, $p < 0.001$) and *H. ligniperda* (binomial GLM: deviance = 262.5, $df = 1$, $p < 0.001$). For both species, all interactions involving fumigation temperature, fumigation and cuticle-coating were not statistically significant ($p > 0.001$). Although cuticle-coating with nail

polish resulted in decreased mortality of both species, as compared with their uncoated controls, significant reductions were found in only *H. ligniperda* (Tables 3.2). No mortalities were recorded for no coating with nail polish and no fumigation with EDN, concerning both species.

Table 3.2 Effect of cuticle-coating and fumigation on the mean mortality (%) of adult *Arhopalus ferus* (at 48 hr) and *Hylurgus ligniperda* (at 96 hr), after 3-hr exposure to ethanedinitrile fumigations (pooled data from 10 and 20 °C).

Treatment	% Mortality (± SE) of	
	A. ferus	*H. ligniperda*
Coated with nail polish, fumigated	28.50 ± 8.00 b	18.00 ± 5.00 b
Uncoated, fumigated	45.00 ± 9.00 b	43.00 ± 6.00 c
Coated with nail polish, not fumigated	0.00 ± 0.01 a	0.00 ± 0.01 a
Uncoated, not fumigated	0.00 ± 0.01 a	0.00 ± 0.01 a

Treatments with the same letter in columns are not significantly different from each other ($p < 0.01$), LSD for 10 individuals per replicate.

3.4 Discussion

Basic information on the presence and functions of morphological features and adaptations of insects to xenobiotic conditions is critical in understanding how pesticides, including fumigants, kill insects and how such information may be used in designing effective control strategies. Therefore, part of my first hypothesis was that differences in the location, structure and function of spiracles on *A. ferus* and *H. ligniperda* adults may contribute to the observed differences in EDN toxicity between these two species.

In line with Ritcher (1969), my findings show the presence of seven abdominal spiracles in both *A. ferus* and *H. ligniperda*. Reduced spiracle number (from eight to seven) has also been reported in many other families of Coleoptera (Chaudhari 2016; Tonapi 1958b). Reduced number of spiracles might indicate redundancy in the contribution of some spiracles for survival, while an increased number may be associated with increased oxygen need. The rate of exchange of gases

in a diffusion-based respiratory system, like those of insects, is known to depend on the area of spiracle openings (Lighton et al. 1993) as larger openings may offer more entry and exit pathways. Therefore, the possession of larger total spiracle sizes for adult *A. ferus* (Fig. 3.5 A) suggests that the species might be able to inhale more EDN fumigant through their spiracles than adult *H. ligniperda*. Hence, the contributions of each spiracle in the exhalation or inhalation of EDN might differ between the two species.

The efficacy of some insecticide classes on insects has been associated with the closeness of these spiracles to the insect brain (Sugiura et al. 2008). Huber (1965) observed that the mesothoracic spiracles could offer rapid entry of gases into insect bodies and provide the quickest route to the CNS. Therefore, the location and morphological features of these spiracles were examined here. The results have shown that, of all spiracles on both species, mesothoracic spiracles were closest to the brain (Fig. 3.4). Further morphological studies on the mesothoracic spiracles revealed that the size, number, shape, height, proximity and orientation of setae covering the openings were different in both beetle species. While the spiracular setae in *A. ferus* are large, branched (bi-pectinate), fewer in number and separated from one another, those of *H. ligniperda* are straight, simple acuminate, small, short and more compact. Sukontason et al. (2006) found similar setae in the mesothoracic spiracles of *M. domestica* and reported that their presence could prevent the entry of dust. Chapman and Chapman (1998) added that, despite the difference in size and orientation of the mesothoracic setae, their primary function was to filter dust and other fine particles. The short, compact and dense setae around the mesothoracic spiracles of *H. ligniperda* may be useful in trapping humid air (Nagpal et al. 2003) and also create conditions for the fumigant to quickly combine with moisture (Emekci 2010) around the spiracular openings. Though this reaction may lead to the break-down of EDN to HCN and other products, some of these break-down products may be unable to successfully pass through the tracheal system to the final target site(s). Furthermore, the presence and contribution of such special setae in maintaining humid conditions around the openings of the mesothoracic spiracle of *H. ligniperda* could cause a faster closure of the spiracles (Nagpal et al. 2003) and thus may limit the uptake of EDN. Therefore, based on the above morphological features, *H. ligniperda* may be more efficient in preventing entry of EDN through the mesothoracic spiracles, compared to *A. ferus*. Considering that the inner closing mechanism in the metathoracic spiracles of *A. ferus* showed large, narrow space inside the atria, but that of *H. ligniperda* comprised both inner

and outer spiracular lids and valves with a smaller size, with less space in the atria, a more effective regulatory control of EDN entry into *H. ligniperda* is possible.

Adult *A. ferus* beetles were significantly larger than *H. ligniperda* (Fig. 3.5 B). The rate of exchange of substances largely depends on the surface-area-to-volume ratio of insects (Kühsel et al. 2017). Large insects possess a smaller surface-area-to-volume ratio. The larger surface area in *A. ferus* means increased exposure to the environment and the vulnerability of body cells to environmental factors including toxic air or fumigant exposure. Therefore, as the surface area of *A. ferus* is proportionally reduced (lower surface-area-to-volume ratio), the demand for uptake of air for gaseous exchange also increases due to the increased number and size of cells. To assess enough oxygen to meet the demands of the numerous cells, large beetles are known to explore multiple respiratory mechanisms such as contraction and relaxation of the thoracic and abdominal body segments (Wasserthal 1996). This is because simple passive diffusion via the tracheal system, which is enough for small insects (e.g., *H. ligniperda*), might be inefficient for larger ones like *A. ferus*. My results show that the main route of entry for EDN into adult *A. ferus* is the thoracic spiracles, suggesting that the abdominal spiracles may be responsible for exhalation of the fumigant from the insect body and thus contraction and relaxation of thoracic and abdominal spiracles might be an important mechanism of fumigant uptake. During active movement, the fatigue rate in *A. ferus* may increase, with higher demand for oxygen (Wigglesworth 1930). This situation coupled with the possession of larger spiracle sizes may compel the insect to inhale EDN more readily than *H. ligniperda*. Though the results agree with Najar-Rodriguez et al. (2015a) that the toxicity of EDN increases at a higher temperature (i.e., higher mortalities for 20 °C), such differences were not significant in the current research. This may be because the toxicity effect of LC_{50} used for the experiments involving both 10 °C and 20 °C were predetermined. Therefore, irrespective of the fumigation temperature, the mortality results were already chosen to represent the LC_{50} required.

In line with Chown et al. (2007b), comparison of tracheal investment and body size of both insects suggests that *A. ferus*, as the larger insect, may have lower mass-specific metabolic and gas exchange rates, which may enhance EDN efficacy. Additionally, the presence of more tracheoles and larger tracheal tubes in *A. ferus* may increase the surface area available for

diffusion and oxygen supply to the cells. Further work on the comparative rates of respiration between both beetles when exposed to EDN is recommended.

Morphological differences were observed in the cuticle thickness and composition between *A. ferus* and *H. ligniperda*. Generally, the cuticle of adult *H. ligniperda* was thicker at the ventral thorax and ventral abdomen, as compared to *A. ferus* (Fig, 3.9). However, the cuticle of *A. ferus* was thicker at the dorsal abdomen and dorsal thorax. Significant differences in individual cuticle layers and total cuticle thicknesses measured from different body parts of both species suggest that cuticle thickness may not be the main factor contributing to EDN tolerance but might be a contributing factor that could reduce cuticular penetration. The resistance of insects to insecticides may be due to delayed penetration of the compound in the insect cuticle (Vinson & Law 1971). The presence of additional chitin layers (Fig, 3.10) in the cuticle of *H. ligniperda* suggests the possibility of delayed/decreased penetration of EDN into this insect, and the likelihood of survival when sub-lethal doses are used. Lin et al. (2012) cautioned that decreased penetration of insecticides through the cuticle, with modifications of the structure or composition of the cuticle in some insects, should be an integral component of resistance management discussions. Further investigations regarding cuticle density, as a physical property of the species and its contribution to the tolerance to EDN by both insect species, are recommended.

The fact that for species, blocking all abdominal spiracles and fumigating resulted in higher mortality, compared to their unblocked and fumigated controls (Table 3.1), suggest that the abdominal spiracles might not be the main route of entry for EDN; but function as the primary exit routes for the fumigant. Additionally, while the mortality of all thoracic spiracles blocked and fumigated adult *H. ligniperda* was not statistically different from the unblocked and fumigated controls, significant differences were found in those for adult *A. ferus* (Table 3.1). The results suggest that the thoracic spiracles might be the main route of entry for EDN into adult *A. ferus*, as blocking and fumigating them led to significantly lower mortality in the beetles. Such a significant reduction in mortality may be due to decreased rate of EDN entry through the abdominal spiracles, as the thoracic spiracles were blocked.

The rate of uptake of vaporized pyrethroid into *M. domestica* was decreased due to the blocking of the mesothoracic spiracle (Sumita et al. 2016). Considering that (i) air entering the insect body

through the mesothoracic spiracles is first channelled to the brain and then to the rest of the CNS (Burrows 1980), and (ii) the thoracic spiracles are closest to the brain, which is the main part of the CNS (Sugiura et al. 2008), the thoracic spiracles might have offered a quicker route of entry for EDN into adult *A. ferus*. This mechanism of respiration is similar to what has been described in *Schistocerca americana* (D.) (Orthoptera: Acrididae) (Burrows 1980; Miller 1960). The 100% mortality observed in all spiracle-blocked and fumigated *A. ferus* and *H. lgniperda* suggest that spiracle-blocking did not prevent the entry of EDN into the insects. Therefore, EDN might have penetrated the insects through other body parts. The penetration of substances into insects through other body parts, despite blocking spiracles, has been observed in some species. For example, oxygen penetrated the larvae of *Hemipyrellia ligurriens* (Diptera: Calliphoridae), even when spiracles were blocked (Fraenkel & Herford 1938). Also, a toxic quantity of MB had accumulated in the larvae of *T. mauritanicus* beetles, despite blocking access to tracheal entry of the fumigant into the insects (Monro 1959). Monro (1959) also reported that some oxygen uptake occurred when spiracles of *Chaerocampa elpenor* (L.) (Lepidoptera: Sphingidae) larvae were blocked with grease, and when *T. molitor* and *Culex sp*. (Diptera: Culicidae) larvae were submerged in water.

The inability of spiracle-blocked insects to recover after EDN fumigation at 48 hr (for *A. ferus*) and 96 hr (for *H. ligniperda*) might be because blocking prevented the escape of the fumigant and prolonged close contact and reaction between the fumigant molecules and the moisturized body cells (Emekci 2010), resulting in insect death. Therefore, conditions that bring fumigant molecules closer to the internal body cells may enable easier diffusion and fumigant efficacy. In related research, Bond (1961a) proved that the rate of uptake of HCN by *S. granarius* depended on the rate of fixation of the fumigant in the insect tissues. Furthermore, the gradual recovery of fumigated insects with unblocked spiracles suggests their capabilities of reducing the toxic effect of EDN by exhalation through the unblocked spiracles.

The current results have shown that though cuticle-coating with nail polish reduced the mortality of both species, suggesting a reduction in fumigant entry due to coating, such reductions were only significant for *H. ligniperda* (Table 3.2). Comparing the high mortality rate when blocking all thoracic spiracles with that when coating the cuticle, the results suggest that the tracheal

system plays a minor role in the overall uptake of EDN into adult *H. ligniperda*. Rather, the cuticle may be regarded as the primary route. The insect cuticle has been cited as the main route of entry for different types of insecticides into insects (Galley 1967; Sumita et al. 2016). Petroleum oils are also known to penetrate the insect cuticle and accumulate in lipid-containing tissues, before entering the nerve cells (Najar-Rodriguez et al. 2008). In addition, contact insecticides are known to first enter insect bodies through the cuticle and proceed to the CNS, using the haemolymph as a medium (Grissom Jr et al. 1989; Matsumura 1963; Yu 2008). Given that (i) the cuticle in *H. ligniperda* possess more chitin layers and is denser than in *A. ferus* (Fig. 3.10 B, C, E, and F), (ii) the cuticle is regarded as the main route of entry into the insect and (iii) the total spiracle area of *H. ligniperda* is comparatively smaller and could offer resistance to the uptake of EDN, delayed penetration of the fumigant through the cuticle of *H. ligniperda* might be an important factor for the observed tolerance of this species. Even though results from the cuticle-coating tests (Table 3.2) have also shown that EDN was less effective in causing a significant reduction in insect mortalities at both 10 °C and 20 °C, mortality due to blocking all thoracic spiracles was higher than that for coating the cuticles with nail polish.

In conclusion, my findings have shown that the possession of smaller spiracle areas, larger body surface-area-to-volume ratio, larger body surface-area-to-spiracle area ratio, denser spiracular setae and generally thicker cuticle with more chitin layers could be morphological characteristics which explain the higher tolerance of adult *H. ligniperda* to EDN. Furthermore, the results have revealed that the cuticle might be the main route of entry for EDN into the body of adult *H. ligniperda* and the thoracic spiracles played a minor role. For adult *A. ferus*, thoracic spiracles may be regarded as the main route of entry for EDN. The outcome of these findings contributes to the general understanding of (i) the existence of species-specific differences to EDN toxicity, (ii) the various mechanisms behind the toxicity of EDN to target insects and (ii) the mode of entry of the fumigant for the effective control of insects. Having identified the main routes of entry for EDN into both species, further work will investigate the behavioural effects as indicators of the fumigant mode of action against the insects.

CHAPTER 4 Evaluation of Behavioural Responses of *Arhopalus ferus* and *Hylurgus ligniperda* adults to Ethanedinitrile

4.1 Introduction

Studies have shown that insects can withstand insecticide exposure through various physiological adaptations or behavioural modifications (Jallow & Hoy 2005; Li et al. 2007). Such varied responses may reflect insecticide mode of action against target insects (Haynes 1988) or greatly increase their survival if the behaviour is modified to minimise or avoid exposure and uptake (Barson et al. 1992; Braga et al. 2011b). Therefore, behavioural modifications may exist in different forms. For example, avoidance behaviour as a strategy to survive insecticide exposure has been reported in some insects (Cox et al. 1997; Wang et al. 2000; Watson & Barson 1996). Also, adult Psocid, *Liposcelis bostrychophila* (Badonnel) (Psocoptera: Liposcelididae) reduced their walking activities to minimise exposure to three commercial insecticides (*β*-cyfluthrin, chlorfenapyr and pyrethrins) (Guedes et al. 2008). Behavioural modifications may sometimes compromise insecticide efficacy tests against insect pests; as a result, standard assays based on lethality may either overestimate or underestimate the impact of insecticides on insects. For instance, effective control of *L. bostrychophila* was compromised because the insects moved away and delayed egg-hatching during phosphine fumigation (Nayak et al. 2003).

Such limitation is particularly important for species that are already physiologically resistant or tolerant. Nevertheless, insecticides can affect insect behaviour, regardless of whether insects were previously resistant or tolerant (Guedes et al. 2008; Guedes et al. 2009a; Watson & Barson 1996; Watson et al. 1997). To determine the effectiveness of insecticides on insect pests, accurate assessment of both behavioural and physiological effects on the overall efficacy is required (Biondi et al. 2012). However, no correlation was shown between behavioural avoidance and physiological resistance when adult of the maize weevil, *Sitophilus zeamais* Motschulsky (Coleoptera: Curculionidae), another EDN-tolerant species, were exposed to insecticides (Braga et al. 2011b; Guedes et al. 2009a). Thus, species-specific adaptations in terms of behavioural avoidance may occur across insects. Till now, just a few studies have investigated how insects change behaviour in response to insecticide exposure and how behavioural

modifications affect the mode of action and toxicity of insecticides. Considering that two of our target insects: *A. ferus* and *H. ligniperda* have demonstrated different levels of tolerance to EDN, it is not known whether any of the insects, particularly the more tolerant *H. ligniperda*, uses any of the above-stated behavioural modifications as a protective mechanism to enhance its chances of survival.

Exposure to insecticides may also induce a narcotic effect in insects (Winks 1984). Narcosis can be described as a state of stupor, drowsiness, unconsciousness and respiratory inhibition in insects after exposure to toxic substances, resulting in temporal immobilization (Nakakita et al. 1974; Semper 1883). Therefore, behavioural changes such as decreased mobility and gradual immobilization may occur at initial stages of narcosis in some insects (Csik 1940). This may be reversed with signs of recovery when insects are re-exposed to normal air conditions (Bond 1963). Narcosis can also provoke initial excitation or walking activity in other insects, as in the case of etherisation or treatment of the fruit fly, *Drosophila melanogaster* (Meigen) (Diptera: Drosophilidae) with CO_2 (Van Dijken et al. 1977). It was reported that exposure to ether produced a long-lasting decrease in locomotor activity of the insects, but CO_2, on the other hand, increased locomotor activity, with comparatively shorter duration. The rate of onset of narcosis is thought to be proportional to the dosage of the toxic compound, and this may be most pronounced in resistant or highly tolerant pest populations (Winks 1985). For example, narcosis was reported to have occurred in insects that were exposed to high doses of phosphine (Bang 1967; Bond et al. 1969; Kashi 1982). Resistant populations of *T. castaneum* have shown signs of narcosis as a protective mechanism against phosphine exposure (Winks 1985), which was thought to reduce fumigant efficacy. Also, when adult *T. castaneum* were exposed to a range of phosphine doses, time to narcosis decreased with higher doses (Winks 1984). The author reported that the use of narcosis by the insects as a protective behaviour, may have led to decreased rates of phosphine uptake.

The resistance of insects to some fumigants has also been associated with a reduced rate of respiration, leading to lower uptake (Nath et al. 2011; Pimentel et al. 2007; Pimentel et al. 2012). Such a reduced rate of respiration and fumigant uptake may be due to a reduction in walking activity and lowered metabolism during exposure. Reduced respiration rate as a means of limiting fumigant uptake has also been demonstrated by some stored-product insects resistant to

phosphine (Pimentel et al. 2007). As most insects are capable of inhaling fumigants through the respiratory system, factors that influence their respiration rates could also affect the uptake of a fumigant (Cotton 1932; Fytizas & Katsoyannos 1979; May 1989). Additionally, the rate of oxygen uptake by insects is related to fumigant toxicity (Bond 1956). Considering that adult *H. ligniperda* possessed smaller spiracle area (Gidiglo et al. 2018) and smaller body surface area (Chapter 3), EDN uptake by the insect through the spiracles may be reduced. Such reduced uptake may reflect the toxicity of the fumigant against the insects and perhaps, alter their behaviour during exposure.

Although some studies have addressed the importance of sub-lethal and lethal responses of insects to insecticides, information on the topic is limited (Nayak et al. 2003; Winks 1985). Earlier works regarding insecticide efficacy on insect pests estimated only the lethality of target insects as a physiological mechanism, which differed from the outcome of follow-up assessments of sub-lethal effects on insects (Desneux et al. 2007; Kongmee et al. 2004; Stark & Banks 2003). Though sub-lethal concentrations may show no apparent mortality in insects (Desneux et al. 2007), they may affect the biology (Nayak et al. 2003; Bond & Upitis 1973; Hobbs & Bond 1989), as well as physiology, demography or behaviour of insects that survive their exposure (Barson et al. 1992; Braga et al. 2011b; Pimentel et al. 2012). This area of inquiry, therefore, offers an opportunity to better understand the mode of action of EDN on key forest insects.

So far, no studies have investigated mechanisms behind the toxicity of EDN to insects, and no attention has been paid to whether the fumigant affects the behaviour of target insects. In this Chapter, I carried out experiments to determine how two key forest insects associated with New Zealand export logs, *A. ferus* and *H. ligniperda* (Pawson et al. 2009), responded to lethal (LC_{99}) and sub-lethal (LC_{25}, LC_{50}, LC_{75}) concentrations of EDN (Table 4.1). In parallel, I measured the effect of the fumigant on their walking behaviour using recently developed tracking software.

4.2 Materials and methods

4.2.1 Insects

Field-collected, non-sexed *A. ferus* adults of mixed ages were used in these experiments. Insects were hand-collected at night from timber processing mills near Nelson, New Zealand, from December to March of 2017 and 2018. To avoid mortality due to aggressive behaviour, groups of less than fifty insects each were placed into 1 L plastic containers, with lids perforated to enable air exchange and moist paper towels to provide humidity during transport. The containers with insects were also kept cool until delivered to the laboratory. In the laboratory, insects were kept at 10 ± 2 °C in the containers until needed for experimental work. Only live, active and undamaged insects were used in the experiments. Before fumigations, each *A. ferus* adult was picked from the container with a pair of forceps (BioQuip Products, Inc., USA) and transferred into a large Petri dish (150 mm x 15 mm) (with filter paper lining the inner base to provide an even walking-surface). Petri dishes were covered with non-tight fitting lids to prevent escape and allow gaseous exchange. Thereafter, *A. ferus* adults were exposed to the fumigant individually in Petri dishes to avoid aggressive encounters.

One to two-month-old adults of *H. ligniperda* were supplied by the insect breeding team at Plant and Food Research, Auckland. The insect colonies were lab-reared according to Clare and George (2016a) and kept on modified artificial bark diet that was developed from the diet (Rogers et al. 2002) for rearing larvae of the endemic huhu beetle, *Prionoplus reticularis* (White) (Coleoptera: Cerambycidae) (Barrington et al. 2015). Insects were kept at 10 °C until needed for the experiments. About twenty insects were kept in each Petri dish with the diet before experiments.

4.2.2 Behavioural bioassays and fumigations with EDN

Fumigations of *A. ferus* and *H. ligniperda* adults (without diet) were carried out in 28-L chambers (Labconco® desiccators, Kansas City, Missouri, USA). Before each experiment, insects from both species were acclimatized at the selected fumigation temperatures (10 °C and 20 °C) for 72 hr. Before fumigation, concentration-response fumigation tests were conducted and the necessary adjustments made to justify the choice of lethal concentrations shown in Table 4.1.

While fumigant concentrations and effects for adult *A. ferus* did not change from those used by Najar-Rodriguez et al. (2015b), the concentrations for *H. ligniperda* were increased to reflect the required standards of LC_{25-99} shown in Table 4.1. Therefore, the LC_{25-75} used for fumigating adult *A. ferus* were calculated from those reported by Najar-Rodriguez et al. (2015b), administered and mortalities counted. For *H. ligniperda*, LC_{25-75} were determined by conducting trial fumigations using a range of concentrations for each temperature (10 °C and 20 °C), and insect mortality was recorded and compared.

Table 4.1 EDN concentrations (g/m^3) used to fumigate burnt pine longhorn beetle, *Arhopalus ferus* and golden-haired bark beetle, *Hylurgus ligniperda* adults at 10 °C and 20 °C for 3 hr. Lethal Concentrations (LC) was derived from Najar-Rodriguez et al. (2015b)

Species	Temp. (°C)	Concentrations (g/m^3)			
		LC25	LC50	LC75	LC99
A. ferus	10	1.6	3.2	4.9	6.5
A. ferus	20	1.4	2.8	4.2	5.6
H. ligniperda	10	75.0	150.0	220.0	300.0
H. ligniperda	20	60.0	120.0	180.0	240.0

Groups of insects (n = 1 per Petri dish per chamber for *A. ferus*) and (n = 10 per Petri dish per chamber for *H. ligniperda*) were fumigated inside a fumigation chamber for 3 hr at either 10 °C or 20 °C. Each Petri dish inside the fumigation chamber was placed on top of a perforated white plastic bucket (212 mm x 285 mm x 245 mm, Fig. 4.1 B) that drew the set-up closer to the glass-top cover of the chamber to enable easy viewing of the dishes during fumigation by the cameras (Fig. 4.1 E). To provide a clear background in each arena, a white laminated manila card (210 mm × 297 mm, Fig. 4.1 C) was placed in-between each dish and bucket (Fig. 4.1 D). Each fumigation trial at either 10 °C or 20 °C comprised the following five treatments: a control (not fumigated) and four others each fumigated with one of the following EDN concentrations: sub-

lethal (LC_{25}, LC_{50} and LC_{75}) or lethal (LC_{99}). EDN concentrations used are listed in Table 4.1. At each temperature, movement of insects in the fumigation chambers (Fig. 4.1 E) during the 3-hr fumigations was recorded continuously in 30 min segments for each replicate (i.e., one insect/Petri dish/chamber for *A. ferus* and 10 insects/Petri dish/chamber for *H. ligniperda*) using five different HDR-CX405 video cameras (Sony Handycam, Tokyo-Japan) with each camera mounted directly above the respective chamber (Fig. 4.1E). The experimental design was 2 x 4 factorial (with time as a repeated factor), with 15 replicates/concentration/temperature for *A. ferus* and 3 replicates/concentration/temperature for *H. ligniperda*. Overall, 150 adult *A. ferus* and 300 *H. ligniperda* were used for the entire experiments.

Figure 4.1 Equipment used to record the behaviour of *Arhopalus ferus* and *Hylurgus ligniperda* adults exposed to sub-lethal and lethal EDN concentrations at 10 °C and 20 °C. (A) prepared Petri dish with one adult *Arhopalus ferus*; (B) perforated plastic bucket and (C) laminated manila card; (D) assembled view; (E) prepared Petri dish with ten adults *Hylurgus ligniperda*; (F) fumigation chambers (Ch1-Ch5) and video cameras (C1-C5) positioned above the chambers to monitor walking activities of insects for the entire 3 hr.

The videos were taken under low-intensity artificial lighting (10.5 W, 220-240 V, 95m A) and digitally transferred to a computer for analysis of movement parameters using *ToxTrac* (Version 2.82; Rodriguez et al. (2017)), an automated video tracking software. Measurements taken included total distance walked (mm), average walking speed (mm/s), exploration rate, i.e., the proportion of the Petri dish area covered by walking and the number of stops, i.e., the number of times the insects stopped moving in the chamber. Since the videos captured 1 adult per Petri dish per chamber for *A. ferus*, *ToxTrac* analyzed the movement parameters for only 1 insect at a time. For *H. ligniperda*, average movement parameters of 10 insects per Petri dish per chamber were analyzed by the software.

4.2.3 Fumigant monitoring

During fumigations, EDN doses in the chambers were monitored by collecting head-space samples at 0 (start of fumigation), 1, 2 and 3 hr (end of fumigation). EDN doses in the treated space of chambers were quantified using the validated method of Brierley et al. (2019). Briefly, quantification was done using an Agilent 7890A GC fitted with a flame ionization detector and a capillary column (30 m x 0.53 mm) GS-Q (Agilent Technologies Inc., Auckland, New Zealand). The oven and front detector temperatures were 150 °C and 300 °C, respectively. Fumigant samples were withdrawn from the chambers using a gas-tight syringe (Valco Instruments Co., Texas, USA). Though 5 ml samples were injected, only 1 ml sub-sample was analyzed. During each day of fumigation, dilutions of pure EDN with air were used to establish a five-point calibration curve. After fumigation, chambers containing the insects were aerated for 30 min (with the aid of a vacuum pump connected to an EDN-scrubbing system - Nordiko Quarantine Systems Pty Ltd, Sydney) to ensure no fumigant remained in the chambers. Insects were then removed from the chambers and further aerated under the fume-hood for 1 hr and then immediately transferred into clean labelled Petri dishes with diet (for *H. ligniperda*) or without diet (for *A. ferus*). Considering that the tolerance of *H. ligniperda* to EDN and the failure of *A. ferus* to recover after EDN fumigations, additional "insect recovery" videos of *H. ligniperda* were taken in a 20 °C temperature-controlled room for 30 min at 2, 4, 8, 12, 24, 48 and 96 hr after fumigation for the analysis of the same mobility parameters.

4.2.4 Statistical analyses

Due to the violation of Gauss-Markov assumptions (such as normality on residuals) on which the linear model was based, data were transformed using Box-Cox transformation on all dependent variables. Because of non-normality of residual data, Box-Cox lambda (0) = log transformation was conducted. Analysis models were then applied to log-transformed data. On a log scale, the variability over time was more consistent, which is a requirement for analysis of variance (ANOVA). For both species, ANOVA based on a generalized least squares method with time (T) as a repeated measure or its equivalent was conducted in R software package (extension-nlme). To overcome heteroskedasticity in each model, data were weighted (weight = Treatment) and adjusted for autocorrelation using an auto-regressive covariate structure. The fixed effects of concentration, temperature, fumigation time for *H. ligniperda,* and a polynomial time effect (Time + Time^2) as a covariate were included in the linear model. In addition, quantification of the magnitude of experimental effect on walking parameters was done by calculating effect sizes, according to Grissom and Kim (2012).

4.3 Results

4.3.1 Walking behaviour in *A. ferus* during fumigation

Sample representative tracks of the typical walking behaviour of fumigated vs non-fumigated adult *A. ferus* in the Petri dish arenas are shown in Fig. 4.2. Adult *A. ferus* that were fumigated with low EDN concentrations had less activity at longer periods, whilst those that were exposed to higher EDN concentrations exhibited high walking speed within relatively shorter periods of exposure (Fig. 4.2, 4.3). Such 'hyperactive' walking speed lasted ≤ 30 min of fumigant exposure, after which drastic reductions occurred.

The results show that fumigation with EDN reduced walking activities in adult *A. ferus* (Fig. 4.3**,** Table 4.2). Though such reductions were similar at both temperatures, the greater reduction occurred at 20 °C, especially with higher EDN concentrations. Higher fumigant concentrations caused greater reductions in walking distance and speed, after about 1.5 hr of fumigation. At initial stages of fumigant exposure, the number of times insects stopped walking and attempted to move again increased at higher EDN concentrations (Fig. 4.3). Of all four walking activities, the number of stops made by the insects during walking (lower) and the rate at which they

explored the Petri dish arena (higher) significantly affected by fumigation temperature (Tem) (Table 4.2). All walking activities were significantly affected by fumigant concentration, with the most significant effect being exploration rate ($\eta^2 = 0.51$). Number of times also significantly affected fumigant exposure time. The interaction between temperature and concentration significantly affected the number of stops made by the insects (low), their exploration rate (low) and walking distance (high). The interactions between concentration and the polynomial time effect (T^2), and between concentration and time significantly affected all walking activities. Likewise, both Tem x C x T and Tem x C x T^2 interactions were significant for walking distance, number of stops and speed.

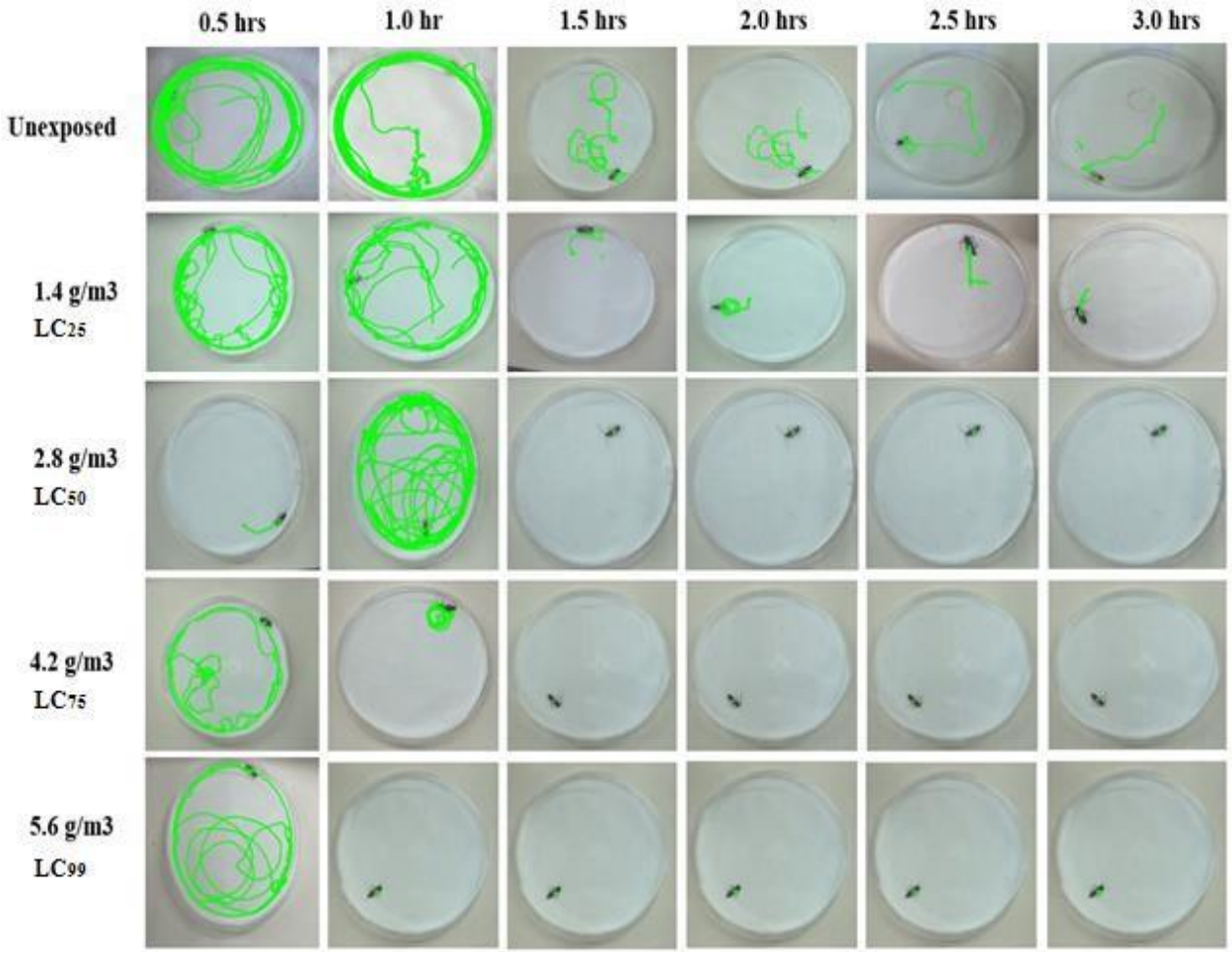

Figure 4.2 Effects of fumigation temperature (10 °C or 20 °C), sub-lethal and lethal ethanedinitrile (EDN) concentrations (g/m^3, see Table 4.1), fumigation time (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hr), and their interactions on total distance walked (mm), walking speed (mm/s), number of stops and exploration rate of adult burnt pine longhorn beetle, *Arhopalus ferus* (Coleoptera: Cerambycidae) during 3 hr exposure to ethanedinitrile.

Table 4.2 Effects of fumigation temperature (10 or 20 °C), sub-lethal and lethal ethanedinitrile (EDN) concentrations (g/m^3, see Table 4.1), fumigation time (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hr), and their interactions on total distance walked (mm), walking speed (mm/s), number of stops and exploration rate of adult burnt pine longhorn beetle, *Arhopalus ferus* (Coleoptera: Cerambycidae) during 3 hr exposure to ethanedinitrile.

Treatment	Mobility parameter	Num DF, Den DF	F - value	*P* - value
Temperature (Tem)	Walking speed	1, 853	1.0454	NS
	Walking distance	1,853	0.9963	NS
	Exploration rate	1,853	12.0677	0.0005*
	Number of stops	1,853	30.773	<0.0001*
EDN conc. (C)	Walking speed	4,853	11.9966	<0.0001*
	Walking distance	4,853	24.7408	<0.0001*
	Exploration rate	4,853	69.0341	<0.0001*
	Number of stops	4,853	62.4260	<0.0001*
Time (T, hr)	Walking speed	1,853	0.2301	NS
	Walking distance	1,853	2.2632	NS
	Exploration rate	1,853	2.7480	NS
	Number of stops	1,853	9.9470	0.0017*
T^2 (hr)	Walking speed	1,853	0.2744	NS
	Walking distance	1,853	1.8958	NS
	Exploration rate	1,853	0.4027	NS
	Number of stops	1,853	3.8030	0.0515*
Tem x T	Walking speed	1,853	0.3454	NS
	Walking distance	1,853	1.3452	NS
	Exploration rate	1,853	3.7499	0.0531*

	Number of stops	1,853	11.6310	0.0007*
Tem x C	Walking speed	4,853	2.1213	NS
	Walking distance	4,853	5.4075	0.0003*
	Exploration rate	4,853	6.0886	<0.0001*
	Number of stops	4,853	10.4810	<0.0001*
Tem x T^2	Walking speed	1,853	0.2999	NS
	Walking distance	1,853	0.9665	NS
	Exploration rate	1,853	1.9441	NS
	Number of stops	1,853	7.7560	0.0055*
C x T	Walking speed	4,853	18.8798	<0.0001*
	Walking distance	4,853	37.2801	<0.0001*
	Exploration rate	4,853	105.7731	<0.0001*
	Number of stops	4,853	104.2700	<0.0001*
C x T^2	Walking speed	4,853	20.5766	<0.0001*
	Walking distance	4,853	33.0133	<0.0001*
	Exploration rate	4,853	80.4164	<0.0001*
	Number of stops	4,853	85.6100	<0.0001*
Tem x C x T	Walking speed	4,853	6.9401	<0.0001*
	Walking distance	4,853	6.6426	<0.0001*
	Exploration rate	4,853	0.9426	NS
	Number of stops	4,853	5.2190	0.0004*
Tem x C x T^2	Walking speed	4,853	13.7397	<0.0001*
	Walking distance	4,853	11.5368	<0.0001*
	Exploration rate	4,853	1.1141	NS

	Number of stops	4,853	3.8060	0.0045*

‘*’ and ‘NS’ indicate significant and not significant figures, respectively, at α = 0.05 (ANOVA). Num DF, numerator degree of freedom; Den DF, denominator degree of freedom.

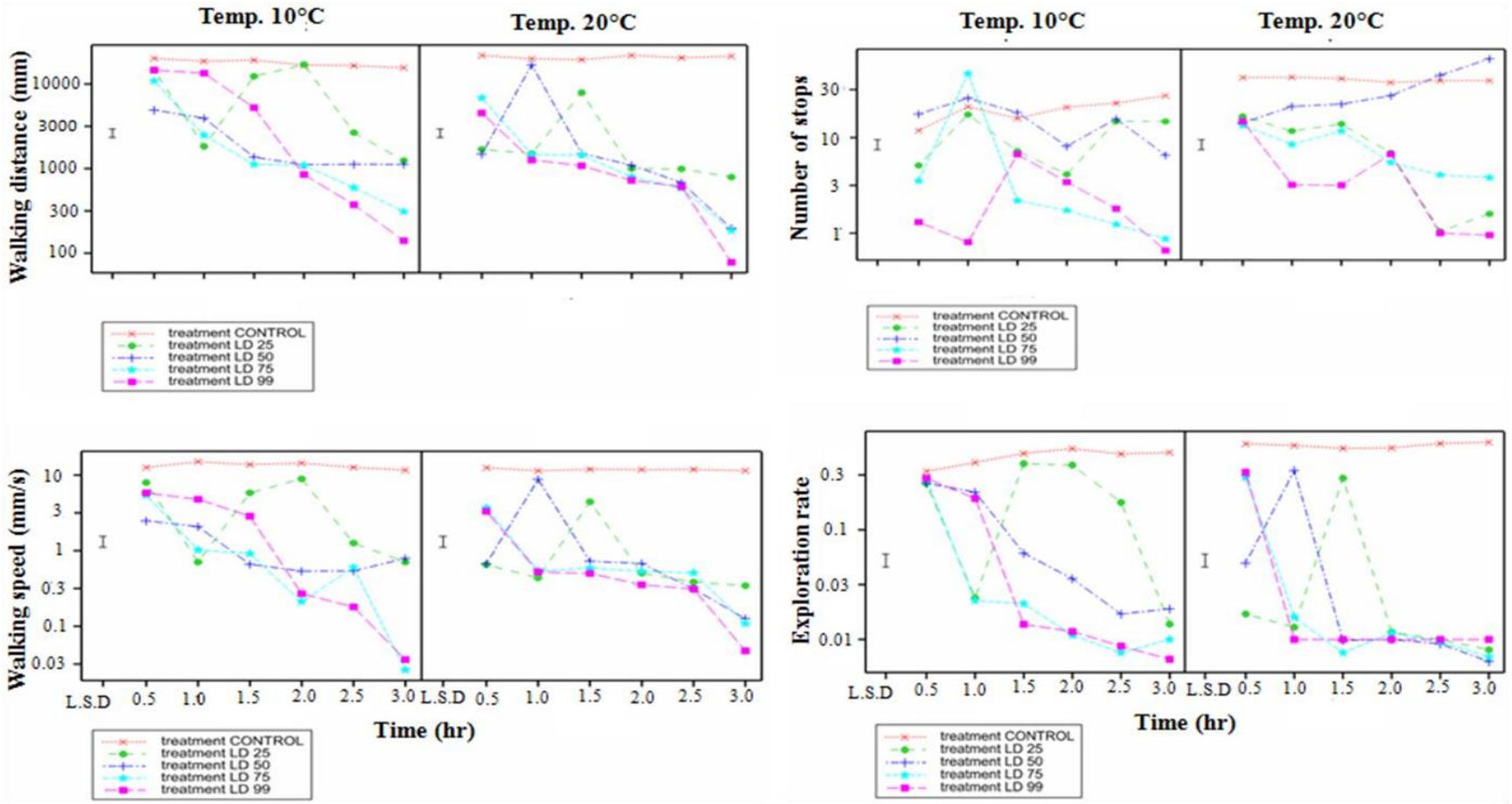

Figure 4.3 Effects of temperature and ethanedinitrile (EDN) concentrations (indicated as LD, i.e., Lethal Dose) on the mean (n = 15) distance walked (mm), walking speed (mm/s), number of stops and exploration rate of adult burnt pine longhorn beetle, *Arhopalus ferus* (Coleoptera: Cerambycidae), during exposure to sub-lethal and lethal EDN concentrations for 3 hr.

4.3.2 Walking behaviour in *H. ligniperda* during fumigation

Sample representative tracks of the typical walking behaviour of fumigated vs non-fumigated adult *H. ligniperda* in the Petri dish arenas are shown in Fig. 4.4. Fumigation caused a reduction in walking activities of adult *H. ligniperda* (Fig. 4.5). Such reductions were similar for both temperatures, but greater reductions occurred at 20 °C, especially with higher EDN concentrations. Except for the controls that were not fumigated, there were instant reductions in insect walking activities (walked distance, exploration rate and speed), at the beginning of all fumigations (Fig. 4.5). Of all factors, only EDN concentration had a significant effect on the exploration rate, a number of times insects stopped walking in the Petri dishes and walking speed, but the latter was higher (Table 4.3). The effect sizes showed that the larger effect of concentration was for the exploration rate ($\eta^2 = 0.41$), rather than for the number of stops ($\eta^2 = 0.11$). The interaction between concentration and time was significant for all walking activities, except for the number of stops. Furthermore, C x T^2 significantly affected all walking activities, except walking speed. The interaction between Tem, C and T had a significant effect on exploration rate and distance walked. The interaction between Tem, C and T^2 also significantly affected walking distance and exploration rate.

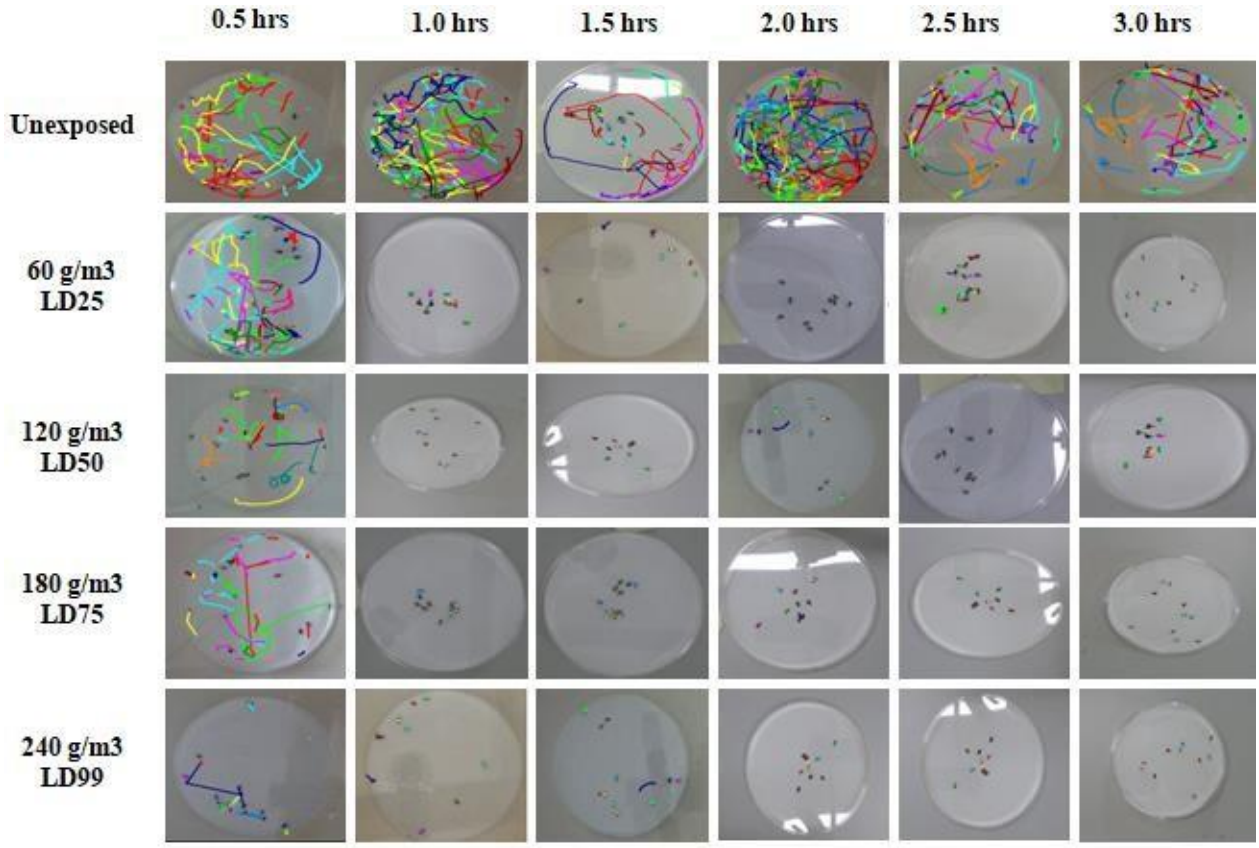

Figure 4.4 Effects of fumigation temperature (10 or 20 °C), sub-lethal and lethal ethanedinitrile (EDN) concentrations (g/m^3, see Table 4.1), fumigation time (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hr), and their interactions on total distance walked (mm), walking speed (mm/s), number of stops and exploration rate of adult golden-haired bark beetle, *Hylurgus ligniperda* (Coleoptera: Circulionidae) during 3 hr exposure to ethanedinitrile. Track colours represent the movement of 10 insects in each Petri dish.

Table 4.3 Effects of fumigation temperature (10 or 20 °C), sub-lethal and lethal ethanedinitrile (EDN) concentrations (g/m3, see Table 4.1), fumigant exposure time (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 hr) and their interactions on walking distance (mm), walking speed (mm/s), the number of stops and exploration rate of adult golden-haired bark beetle, *Hylurgus ligniperda* (Coleoptera: Curculionidae) during 3 hr exposure to ethanedinitrile.

Treatment	Mobility parameter	Num DF, Den DF	F - value	P - value
Temperature (Tem)	Walking speed	1,328	3.4471	NS
	Walking distance	1,328	0.0158	NS
	Exploration rate	1,328	0.2826	NS
	Number of stops	1,328	0.5318	NS
EDN conc. (C)	Walking speed	4,328	4.1913	0.0030*
	Walking distance	4,328	0.7371	NS
	Exploration rate	4,328	9.9565	<0.0001*
	Number of stops	4,328	18.6728	<0.0001*
Time (T, hr)	Walking speed	1,328	0.0604	NS
	Walking distance	1,328	0.1612	NS
	Exploration rate	1,328	0.0018	NS
	Number of stops	1,328	0.0465	NS
T^2 (hr)	Walking speed	1,328	0.0692	NS
	Walking distance	1,328	0.1755	NS
	Exploration rate	1,328	0.0028	NS
	Number of stops	1,328	0.2138	NS
Tem x T	Walking speed	1,328	0.0461	NS
	Walking distance	1,328	0.5290	NS
	Exploration rate	1,328	0.0614	NS

	Number of stops	1,328	0.0046	NS
Tem x C	Walking speed	4,328	3.2057	0.0147*
	Walking distance	4,328	6.3341	0.0001*
	Exploration rate	4,328	4.7466	0.0012*
	Number of stops	4,328	0.8823	NS
Tem x T^2	Walking speed	1,328	0.0413	NS
	Walking distance	1,328	1.7843	NS
	Exploration rate	1,328	0.0336	NS
	Number of stops	1,328	0.0601	NS
C x T	Walking speed	4,328	7.1722	<0.0001*
	Walking distance	4,328	7.1722	<0.0001*
	Exploration rate	4,328	17.7265	<0.0001*
	Number of stops	4,328	0.8823	NS
C x T^2	Walking speed	4,328	4.8002	0.0011*
	Walking distance	4,328	9.6655	<0.0001*
	Exploration rate	4,328	18.6743	<0.0001*
	Number of stops	4,328	6.8308	<0.0001*
Tem x C x T	Walking speed	4,328	2.7467	0.0305*
	Walking distance	4,328	10.6301	<0.0001*
	Exploration rate	4,328	9.9648	<0.0001*
	Number of stops	4,328	0.7719	NS
Tem x C x T^2	Walking speed	4,328	2.1417	NS
	Walking distance	4,328	11.3111	<0.0001*
	Exploration rate	4,328	10.9084	<0.0001*

	Number of stops	4,328	0.9541	NS

‘*’ and ‘NS’ indicate significant and not significant figures, respectively, at α = 0.05 (ANOVA). Num DF, numerator degree of freedom; Den DF, denominator degree of freedom.

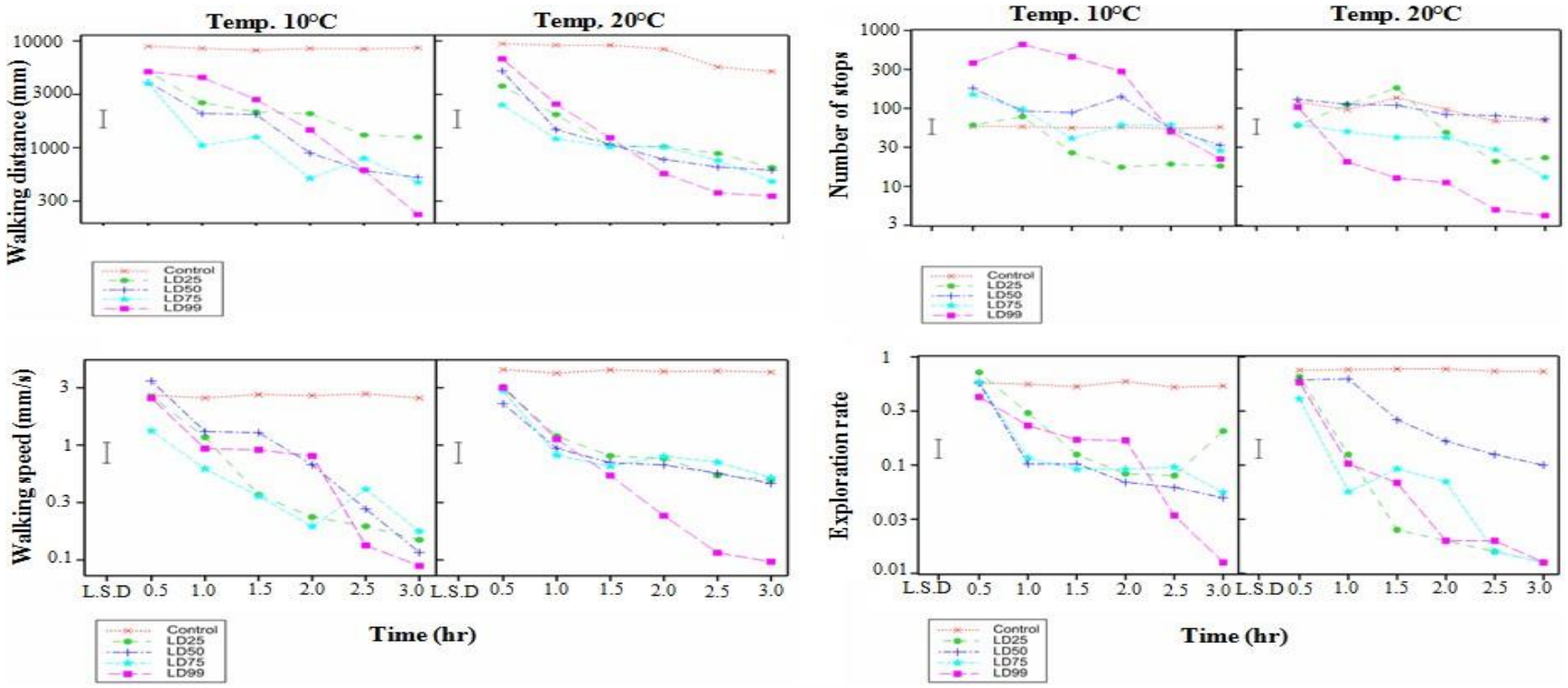

Figure 4.5 Effects of temperature and ethanedinitrile (EDN) concentration (indicated as LD, i.e., Lethal Dose) on mean (n = 10) distance walked (mm), walking speed (mm/s), number of stops and exploration rate of the golden-haired bark beetle, *Hylurgus ligniperda* (Coleoptera: Curculionidae) adults, during exposure to sub-lethal and lethal EDN concentrations for 3 hr.

4.3.3 Walking behaviour in *H. ligniperda* adults recovering from fumigation

After exposure to sub-lethal and lethal EDN concentrations, fumigated insects resumed their movement (Fig. 4.6). From the beginning of recovery, low walking activities were generally observed, but increased activity patterns could be seen towards the end. At both temperatures, insects that were exposed to the lowest concentrations (LC_{25}) exhibited signs of faster recovery (highest exploration rates, number of stops and distance walked) than those exposed to the higher concentrations. As shown in Table 4.4, concentration had a significant effect on all walking activities. The effect sizes show that the most significant effect was on walking distance ($\eta^2 = 0.90$), followed by walking speed ($\eta^2 = 0.89$). Temperature and the (Tem x C interaction had a significant effect on walking distance. The effect of C x T and C x T^2 were significant for exploration rate and the number of stops, while Tem x C x T^2 was only significant for exploration rate.

Table 4.4 Effects of fumigation temperatures (10 and 20 °C), sub-lethal and lethal ethanedinitrile (EDN) concentrations (g/m3, Table 4.1) and recovery times (2, 4, 8, 12, 24, 48 and 96 hr), and their interactions on walking distance (mm), walking speed (mm/s), the number of stops and exploration rate of adult golden-haired bark beetle, *Hylurgus ligniperda* (Coleoptera: Curculionidae) during recovery after 3-hr EDN fumigation.

Treatment	Mobility parameter	Num DF, Den	F - value	*P* - value
Temperature (Tem)	Walking speed	1,328	0.7889	NS
	Walking distance	1,328	15.059	<0.0001*
	Exploration rate	1,328	0.4074	NS
	Number of stops	1,328	0.5318	NS
EDN conc. (C)	Walking speed	4,328	84.8531	<0.0001*
	Walking distance	4,328	111.9080	<0.0001*
	Exploration rate	4,328	155.3290	<0.0001*
	Number of stops	4,328	18.6728	<0.0001*
Time (T, hr)	Walking speed	1,328	1.3442	NS
	Walking distance	1,328	1.349	NS
	Exploration rate	1,328	3.4163	NS
	Number of stops	1,328	0.0465	NS
T^2 (hr)	Walking speed	1,328	1.1919	NS
	Walking distance	1,328	0.813	NS
	Exploration rate	1,328	3.4702	NS
	Number of stops	1,328	0.2138	NS
Tem x T	Walking speed	1,328	0.0368	NS
	Walking distance	1,328	10.537	0.0014*
	Exploration rate	1,328	2.1803	NS
	Number of stops	1,328	0.0046	NS
Tem x C	Walking speed	4,328	4.0582	0.0036*
	Walking distance	4,328	10.5530	<0.0001*
	Exploration rate	4,328	4.1041	0.0033*
	Number of stops	4,328	0.8823	NS
Tem x T^2	Walking speed	1,328	0.1393	NS
	Walking distance	1,328	1.9441	0.0039*
	Exploration rate	1,328	1.8044	NS
	Number of stops	1,328	0.0601	NS
C x T	Walking speed	4,328	3.0211	0.0192*
	Walking distance	4,328	3.5760	0.0078*
	Exploration rate	4,328	8.0027	<0.0001*
	Number of stops	4,328	8.8124	<0.0001*
C x T^2	Walking speed	4,328	1.4716	NS
	Walking distance	4,328	2.3530	0.0557
	Exploration rate	4,328	8.0365	<0.0001*
	Number of stops	4,328	6.8308	<0.0001*
Tem x C x T	Walking speed	4,328	3.158	0.0154*

	Walking distance	4,328	6.0630	0.0001*
	Exploration rate	4,328	5.6198	0.0003*
	Number of stops	4,328	0.7719	NS
Tem x C x T^2	Walking speed	4,328	1.3367	NS
	Walking distance	4,328	3.3930	0.0105*
	Exploration rate	4,328	7.4332	<0.0001*
	Number of stops	4,328	0.9541	NS

'*' and 'NS' indicate significant and not significant figures, respectively, at α = 0.05 (ANOVA). Num DF, numerator degree of freedom; Den DF, denominator degree of freedom.

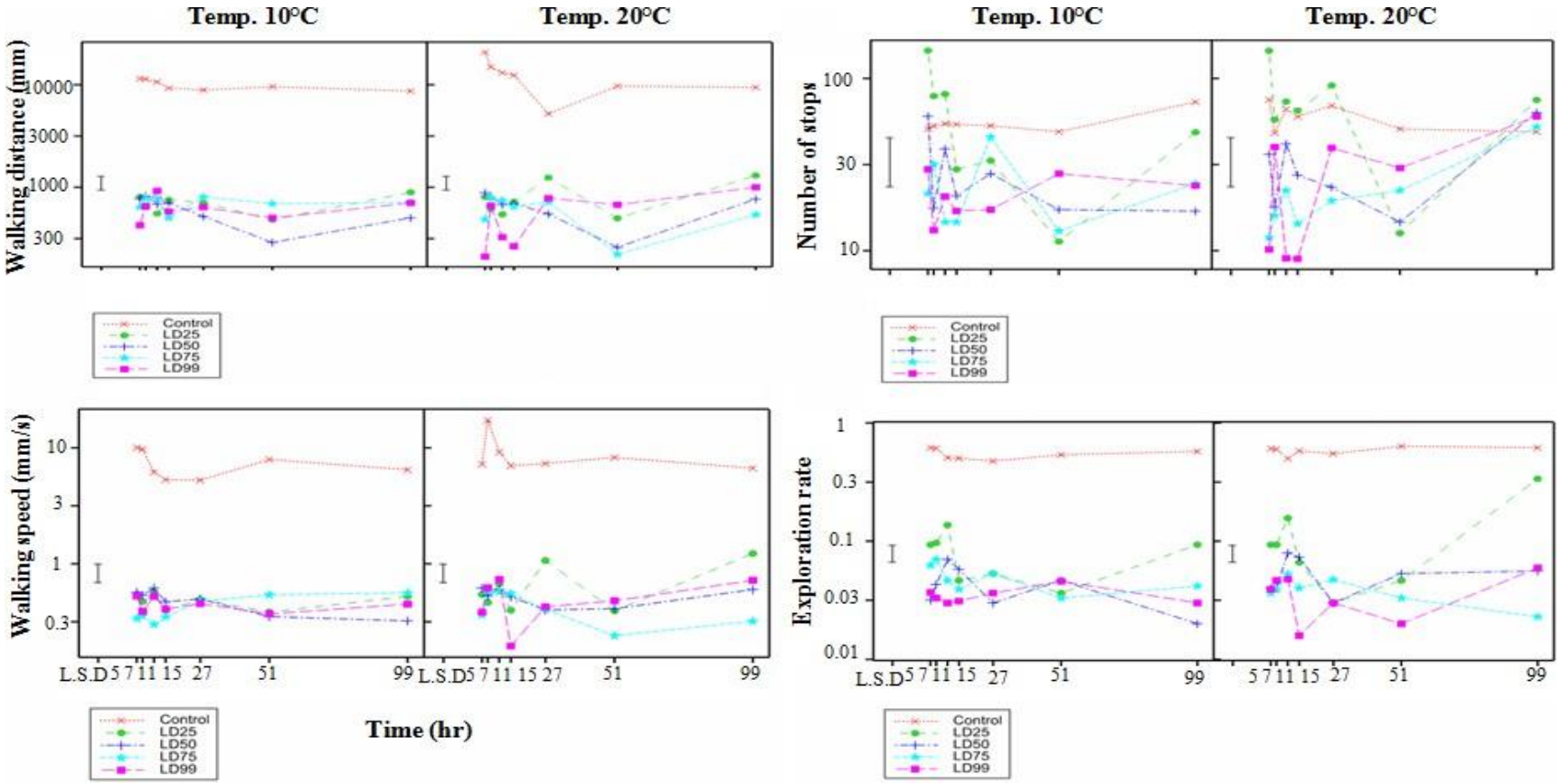

Figure 4.6 Effects of temperature and ethanedinitrile (EDN) concentration (indicated as LD, i.e., Lethal Dose) on mean (n = 10) walking distance (mm), walking speed (mm/s), number of stops and exploration rate of the golden-haired bark beetle, *Hylurgus ligniperda* (Coleoptera: Curculionidae) adults recovering from fumigation with sub-lethal and lethal concentrations at 2, 4, 8, 12, 24, 48 and 96 hr.

4.3.4 Comparisons of walking activities between the two species

EDN exposure reduced the walking activities of both species, with adult *H. ligniperda* showing instant reductions upon its encounter with the fumigant (Fig. 4.4, 4.5), whilst some adult *A. ferus* individuals had increased walking activities at certain points within the fumigation period (Fig. 4.2, 4.3).

Greater reductions in walking activities occurred with increased EDN exposure time and at higher concentrations. *H. ligniperda* showed a higher number of stops when exposed to the 300 g/m^3 (highest dose for LD_{99} at 10 °C). Both species have shown different symptoms of narcosis, with adult *H. ligniperda* reducing walking activities more quickly, as soon as EDN was injected into the fumigation chamber. In comparison, *A. ferus* became progressively inactive a few minutes after showing hyperactivity at certain times during exposure. Before becoming inactive, insects could be seen rolling over on their dorsal side, several times, especially at higher EDN concentrations. At lower concentrations, the above behaviours were not pronounced, and insects were mostly seen adopting normal posture but unable or unwilling to move. In both species, time to narcosis appeared shorter at higher EDN concentrations. During fumigations, fumigant concentration had the most significant effect on the exploration rates of both species.

4.4 Discussion

The behavioural response of insects to insecticides has been a subject of attention in recent years because it may favour the survival of some species (Pimentel et al. 2012; Kaur et al. 2013; Sousa et al. 2012), particularly species that have already shown physiological resistance/tolerance. The current study is the first report on the behavioural responses of insects to sub-lethal and lethal exposures of EDN. Considering that *A. ferus* and *H. ligniperda* differ in their tolerance to EDN, with the former being highly susceptible (Najar-Rodriguez et al. 2015a; Pranamornkith et al. 2014b), while the latter is highly tolerant (Najar-Rodriguez et al. unpublished data), my first hypothesis was that exposure to EDN would result in reduced walking activity as a protective behaviour in *H. ligniperda*. This would possibly decrease the rates of metabolism and respiration and eventually minimise the fumigant uptake by both insects. The present results support this

hypothesis, as adult *H. ligniperda* demonstrated an instant reduction in walking activity, which could be an important factor compromising the efficacy of EDN at controlling this species.

During the 3-hr exposures to sub-lethal and lethal EDN concentrations, EDN caused quicker and instant reduction in the walking activities of adult *H. ligniperda* (Fig. 4.5) but the insects were seen gradually recovering hours after fumigation. In contrast, *A. ferus* adults showed hyper walking activities or hyperactivity at certain points (between 0-2 hr of fumigation) during their fumigation processes (Fig. 4.3). The occurrence of greater reductions in walking activities for adult *H. ligniperda* at 20 °C, especially with higher EDN concentrations, suggests that the toxic effect is greater at higher temperatures and fumigant concentrations. The sudden rise in walking activities by *A. ferus* suggests a very quick toxic effect of EDN on the insects. Reduction in walking activity, as a strategy to minimize and survive insecticide exposure has been reported in some insects (Wang et al. 2000; Cox et al. 1997; Watson et al. 1997). The resistance of *L. bostrychophila* species to pyrethrin insecticide was explained by the insects' ability to reduce walking activity, which likely led to decreased rates of metabolism and respiration; hence, lowered uptake of the compound (Guedes et al. 2008; Pimentel et al. 2007; Pimentel et al. 2008).

Given that higher concentrations and longer exposure times caused further reductions in walking activities (Figs. 4.3 and 4.5), differences in the response between the two species might be attributed to (i) dosage differences, (ii) their relative ability to exclude the fumigant from the site of paralytic action or (iii) differences in resistance of the action site (see Chapter 5). The significant effect of EDN concentration on all walking activities aligns with earlier results of Najar-Rodriguez et al. (2015b) and Pranamornkith et al. (2014c) that adult *A. ferus* are highly susceptible to EDN fumigations. My earlier studies have revealed that spiracles of adult *H. ligniperda* are smaller and less likely to allow more fumigant uptake compared to those of *A. ferus* (Gidiglo et al. 2018). Furthermore, the spiracles of *H. ligniperda* have well-developed and more efficient closing mechanisms than those of *A. ferus* (Chapter 3). Also, *H. ligniperda* possesses more cuticle/chitin layers, suggesting the possibility of delayed penetration of EDN. The results of a follow-up experiment have also shown that the main route of entry for EDN into the species was the cuticle. These findings suggest that adult *H. ligniperda* has both physiological and behavioural advantages that might make it more efficient at preventing the entry of large volumes of EDN into its body, hence could lessen the toxic action of the fumigant.

The combination of both small spiracle area (less fumigant uptake and toxic effect) and reduced walking activity (which could lead to lower respiration and metabolism rates) could be one of the reasons why *H. ligniperda* is more tolerant to EDN than *A. ferus*. Therefore, as suggested by Biondi et al. (2012) for other species, effective control of adult *H. ligniperda* should involve accurate assessment of both behavioural and physiological effects on the overall efficacy.

Behavioural responses of insects to insecticides may reflect the compound modes of action and the extent to which they affect their behaviour (Hoy et al. 1998; Haynes 1988). EDN mode of action against adult *A. ferus* seems to involve temporal stimulation of the respiration rate (indirectly assumed based on observed hyperactivity), leading to increased metabolic rates, followed by paralysis and death. Considering that *A. ferus* has larger spiracle sizes (Gidiglo et al. 2018), coupled with the results that the main route of entry for EDN into the insect for this species might be the spiracles (Chapter 3), the toxic action of EDN might have occurred more effectively at the time when insects were hyperactive (when respiration and metabolic rates were high) as a result of the stimulation. Thereafter, a sharp decrease in peak activity was observed (Figs. 4.3 and 4.5). Likewise, sub-lethal and lethal concentrations of ethylene dichloride and carbon tetrachloride stimulated the respiration rates of *T. castaneum* adults and larvae within a few hours of exposure (Bang 1967).

For adult *H. ligniperda*, the trend in recovery after fumigation was different from that of exposure for the same species. The results have shown that exposure to sub-lethal EDN concentrations might have led to partial respiratory inhibition, as insects gradually recovered hours after fumigation (Fig. 4.6). Bond (1963) reported similar results for *S. granarius* and *T. mauritanicus* when the insects were exposed to HCN, a principal decomposition product of EDN. This effect is similar to the reported "protective stupefaction" (Lindgren 1938) for HCN (an integral component of EDN) that caused complete and rapid respiratory inhibition in adult *S. granarius* but partial inhibition in *T. molitor* (Bond 1959). A partial respiratory inhibition from cyanide has also been reported in *T. molitor* (Bond 1961a), *Curpocupsa pomonella* (Linnaeus) (Lepidopteran: Tortricidae) (Graham 1946) and *Garterophilus intestinalis* (De Geer) (Diptera: Oestridae) (Levenbook 1951).

Alternatively, insects might close their spiracles or stop breathing as protective mechanisms.

Such a situation could enable them to tolerate higher than normal doses of any toxic gaseous molecule. This assumption is supported by past studies. For instance, the resistance of *A. aurantii* to sub-lethal cyanide exposure was associated with the insect's ability to close their spiracles for longer periods during fumigation, whilst the susceptible species could just close them for a shorter time (Hardman & Craig 1941). Unfortunately, this was not tested in the present work due to time limitation and lack of equipment. Although the protective effect in adult *H. ligniperda* may be associated with paralysis and spiracle closure, oxygen deficiency and inhibition of metabolism may also be involved. The rapid paralysis exhibited by *H. ligniperda* right from the onset of fumigation may have reduced the demand for oxygen by the insect cells, and such decreased oxygen requirements may have complemented the anoxic effects of the fumigant. As a recommendation for future work, the respiratory rates of both species during and after EDN fumigation should be measured to establish the relationship between reduced respiration and insect survival.

The undulating trends of walking activities in adult *H. ligniperda* during and after fumigation suggest that EDN exposure caused narcosis in this species. Indeed, time to narcosis was concentration-dependent, with shorter narcosis occurring at higher doses. The phenomenon of insects becoming narcotized when exposed to high fumigant concentrations was previously reported (Bang 1967; Kashi 1982; Nakakita et al. 1974). Fumigant concentration seems to have greatly determined the rate at which both *A. ferus* and *H. ligniperda* responded to EDN (Table 4.2, 4.3, 4.4), as well as time to narcosis. Bang (1967) and Bond et al. (1969) also observed that the rate of narcosis was proportional to the fumigant concentration. This suggests that the rate of uptake of EDN by insects exposed to higher concentrations may be lower, with increased time to narcosis. The situation might explain variations in mortality response of adult *H. ligniperda* at concentrations greater than 200 g/m^3 (Najar-Rodriguez et al. unpublished data) and the currently observed ones at 240 and 300 g/m^3 (Table 4.1), for both 20 and 10 °C, respectively. Regarding narcosis as a protective mechanism, it is possible to predict, based on the results described here that adult *H. ligniperda,* which have shown greater susceptibility to narcosis, may also have greater chances of survival. In addition, *H. ligniperda* showed a higher number of stops when exposed to the 300 g/m^3 (highest dose for LC_{99} at 10°C). The fast recovery of insects that were exposed to lower concentrations at both temperatures suggests that low EDN concentrations had less effect on insects. The rise in walking activities shown by *H. ligniperda* at 96 hr after

fumigation implies that by that time onwards, insects may fully recover. As shown by the results, EDN concentration had significant effect on all walking activities of *A. ferus* during exposure and all, except the walking distance of *H. ligniperda* during exposure. This suggests that concentration may be the main determinant of EDN toxicity in *A. ferus*. Therefore, even though effective control of adult *A. ferus* may be achieved by increasing EDN concentration, that of adult *H. ligniperda* may be more involving. The insignificant effect of concentration on walking distance covered by *H. ligniperda* during EDN fumigation, may explain why the insects tolerate EDN fumigations even at higher doses.

In conclusion, exposure of both species to all EDN concentrations led to decreased walking activities. Such reduced walking activity is likely to lower the respiration rate of the insects, thereby minimising their uptake of EDN. During fumigations, fumigant concentration had the most significant effect on the exploration rates of both species. EDN exposure at higher concentrations induced narcotic responses (similar to "knock-down", with insects falling off several times in the arena) in both species, but this was more pronounced in *H. ligniperda*. In *A. ferus*, EDN fumigation led to an initial excitation, followed by instant paralysis and reduction in walking activities. In *H. ligniperda*, the fumigant first caused instant paralysis, followed by a reduction in walking activities. After the fumigations, *H. ligniperda* exhibited a gradual recovery, suggesting the occurrence of partial respiratory inhibition and the possibility of narcosis being used as a protective mechanism by this species. This could be part of a mechanism that allows *H. ligniperda* to withstand exposure to higher concentrations of EDN more efficiently than *A. ferus.*

CHAPTER 5 Detection of Cyanide in selected Organs of the Burnt Pine Longhorn Beetle, *Arhopalus ferus,* and the Golden-haired Bark Beetle, *Hylurgus Ligniperda*

5.1 Introduction

EDN penetrates quickly through wood (Ren et al. 1997), with a threshold limit value (TLV) of 10 ppm as a comparative advantage against MB (5 ppm) and phosphine (0.3 ppm) (Ren et al. 2006). It is water-soluble (Emekci 2010) and thought to break down to HCN, CO_2 and ammonia (NH_3) in water and high humidity environments (O'Brien et al. 1999). However, when *P. koraiensis* logs were fumigated with the fumigant, Park et al. (2014) did not detect HCN in the treated space. Similarly, EDN was not significantly converted to HCN during the fumigation of *P. radiata* logs (Hall et al. 2018b). The results suggest that either (i) the assumed decomposition of EDN to HCN does not take place during the fumigation or (ii) the HCN produced after EDN breakdown is too negligible to be detected. There are no reports on the break-down of EDN to HCN within the body of target insects.

EDN mode of action against insects is believed to involve hydrolysis to HCN, whereby cyanide ions (CN^-) bind to the iron atom of cytochrome c oxidase (COX) enzyme in the mitochondria of insect cells (Hamel 2011). This prevents the cells, particularly those with high oxygen demand, from having access to oxygen (Anseeuw et al. 2013), causing "cellular asphyxiation" and eventual death. Hydrogen cyanide has also been shown to inhibit the activity of the catalase enzyme in *S. granarius* (Bond 1961b). However, a recent study by Ramadan et al. (2020) on beetles suggests that COX may not be the main target for EDN toxicity. Therefore, the toxic action of CN^- on insects may not be entirely attributed to the inhibition of COX.

The CNS, in conjunction with the endocrine system, controls many life functions and processes in an insect body (Gullan & Cranston 2014). Some toxic substances penetrate the tracheal system or the cuticle into the insect body and once inside migrate to the CNS which is made up of the brain, suboesophageal ganglion, and the ventral nerve cord that extends backwards from the suboesophageal ganglion (Johnson & Triplehorn 2004; Najar-Rodriguez et al. 2008). The ganglia of the insect CNS serves as coordination centre, with each ganglion coordinating

impulses in specified parts of the insect body (Triplehorn & Johnson 2005). Each ganglion has a network of numerous nerve cells: motor neurons and association neurons (Hess 1958; Volkenhoff et al. 2015) densely packed in a neuropile and surrounded by glia cells, which are also enclosed in a neural lamella (Witthöft 1967). Energy requirements and metabolism in the CNS are regarded as complex in terms of the cellular uptake (Lutas & Yellen 2013; Magistretti 2006; Volkenhoff et al. 2015). Therefore, an understanding of how insect organs interact with CN^- is of critical importance to understanding EDN's toxicity and final target site.

One of the enzymes that detoxify CN^- in the insect gut and other parts of the body is *β*-cyanoalanine synthase (Duffey & Blum 1977; Witthohn & Naumann 1987). In southern armyworm, *Spodoptera eridania* (Stoll) (Lepidoptera: Noctuide) and the cabbage looper, *Trichoplusia ni* (Hubner) (Lepidoptera: Noctuidae), *β*-cyanoalanine synthase activity takes place in the mitochondria (Meyers & Ahmad 1991) while in the grasshopper, *Zonocerus variegatus* (Linnaeus) (Orthoptera: Pyrgomorphidae) (Ogunlabi & Agboola 2007) and the larvae of cabbage white butterfly, *Pieris rapae* (Linnaeus) (Lepidoptera: Pieridae) (van Ohlen et al. 2016), *β*-cyanoalanine synthase was found in the gut.

Given that the toxicity of EDN is likely determined by the action of CN^-, the fate of this ion within insect bodies may provide clues with regards to EDN mode of action. I hypothesized that the toxic action of CN^- against insects might relate to its accumulation in any or some of the selected insect organs. The current study investigated the possibility of CN^- accumulation in the gut, fat bodies and ganglia of *A. ferus* and *H. ligniperda* adults, using Attenuated Total Reflection - Fourier Transformed Infrared (ATR - FTIR) technique.

5.2 Materials and Methods

5.2.1 Chemicals

Even though sodium cyanide and potassium thiocyanate were also tested as EDN/HCN surrogate compounds for the presence of CN^-, only Methyl cyanide (CH_3CN) (HPLC grade, 99.9%) was chosen as the most suitable compound for this work, because it produced very sharp CN^- peaks. The compound was obtained from SDS Votre Partenaire Chimie, France. Phosphate buffered

saline (pH 7.4) was obtained from the Entomology laboratory of Plant and Food Research, Palmerston North, New Zealand.

5.2.2 Test Insects

Field-collected, mixed *A. ferus* adults of mixed ages were used in these experiments. Insects were hand-collected at night from timber-processing mills near Nelson, New Zealand in December 2019. To avoid mortality due to aggressive behaviour, groups of less than fifty insects each were placed in 1 L plastic containers (lids perforated to enable air exchange) lined with moist paper towels to provide humidity during transport. The containers were also kept cool until delivered to the laboratory. Once in the laboratory, insects were kept at 10 ± 2 °C in the 1 L plastic containers until needed for experimental work. Only live, active and undamaged insects were used in the experiments. Prior to exposure to methyl cyanide, each insect was picked from the container with forceps (BioQuip Products, Inc., USA).

One- to two-months old first filial generation (F1) adult *H. ligniperda* that were used for these experiments, were obtained from artificially-infested *P. radiata* logs (approx. 25 cm long and 25 cm wide). The parent colony of adults was raised in the laboratory according to Clare and George (2016b) and kept on modified artificial bark diet that was developed, based on Rogers et al. (2002) for rearing larvae of *P. reticularis* (Barrington et al. 2015). Once infested with the parent adults (male to female ratio, 15:15), the logs were enclosed in black plastic bags that were covered with some amount of trapped air, and kept at 20 °C (Fig. 5.1). One end of each log was wrapped with a moist paper towel to provide humidity. Every fortnight, the bags were opened to provide the logs with fresh air. Mating, fertilization, egg-laying, larval development and pupation took place within the logs. Emerged adults were removed from the infested logs by stripping off the bark with a screwdriver, and were immediately transferred to artificial diet in covered Petri dishes (87 mm and 90 mm diameter × 15 mm depth) at 10 °C until needed for experiments. About 20 insects were kept in each Petri dish with the diet.

Figure 5.1 Breeding of adult *Hylurgus ligniperda* on *Pinus radiata* logs. Images taken during the breeding program at Plant and Food Research Institute, Palmerston North, New Zealand.

5.2.3 Insect exposure to methyl cyanide

Before exposure to CH_3CN, all insects were removed from the 10 °C refrigerator and placed in different containers. To avoid aggressive encounters, *A. ferus* adults were placed individually in plastic vented queen bee cages (8 cm × 3 cm × 1 cm, supplied by Waireka Honey, Palmerston North). Adult *H. ligniperda* were kept in the Petri dishes. The insects were held in their cages/dishes for 2 hr (Najar-Rodriguez et al. 2015b) to allow them to acclimate to the treatment temperature (10 or 20 °C). They were then exposed to methyl cyanide (CH_3CN) (675270 g/m^3) soaked in a cotton ball in their cages or Petri dishes and placed in an air-tight plastic container (10 cm × 6 cm × 8 cm, supplied by Thermo Fisher Scientific, East Tamaki, Auckland, New Zealand).

A range of dose-response tests were performed to evaluate the toxicity of CH_3CN and to determine the duration of exposure that could cause complete mortality in both insect species. During the tests, insects were randomly divided into four groups of six insects each, and treated as follows: Group 1: unexposed, Group II: exposed to CH_3CN (35540.55 g/m^3), Group III: exposed to CH_3CN (177703 g/m^3), and Group IV: exposed to CH_3CN (675270 g/m^3).

I first conducted preliminary tests on target insects (20 individuals per species) with EDN using the ATR-FTIR technique (explained below) but detected no traces of CN^- in the insect bodies. Thus, I selected CH_3CN (675270 g/m^3) for this study because it was the only concentration with noticeable CN^- peaks in ATIR-FTIR spectra (Fig. 5.4). Treated insects were exposed to CH_3CN for 3 hr. Control insects were left unexposed and kept in separate containers for the same duration. After exposure, dead exposed insects were transferred into separate containers in preparation for the microscopy work. Live and unexposed ones were immediately anaesthetized at -20 °C for 10 min also in preparation for the microscopy work.

5.2.4 Insect dissection and sample preparation

Insect dissection was done according to Stangier et al. (1989). Briefly, I dissected and isolated the gut (Fig 5.2 B), CNS ganglia (consisting the brain, CNS ganglia and interganglionic connectives) (Fig 5.2 C) and fat bodies (Fig 5.2 B) of twenty individuals from each species that were (i) exposed to CH_3CN (675270 g/m3) and (ii) the unexposed controls. The dissected organs were then quickly collected and placed in clean 1.5-ml Eppendorf tube containing 3 ml phosphate buffer saline (PbS) solution at room temperature. The obtained organs suspensions were then ready for ATR-FTIR spectral analysis.

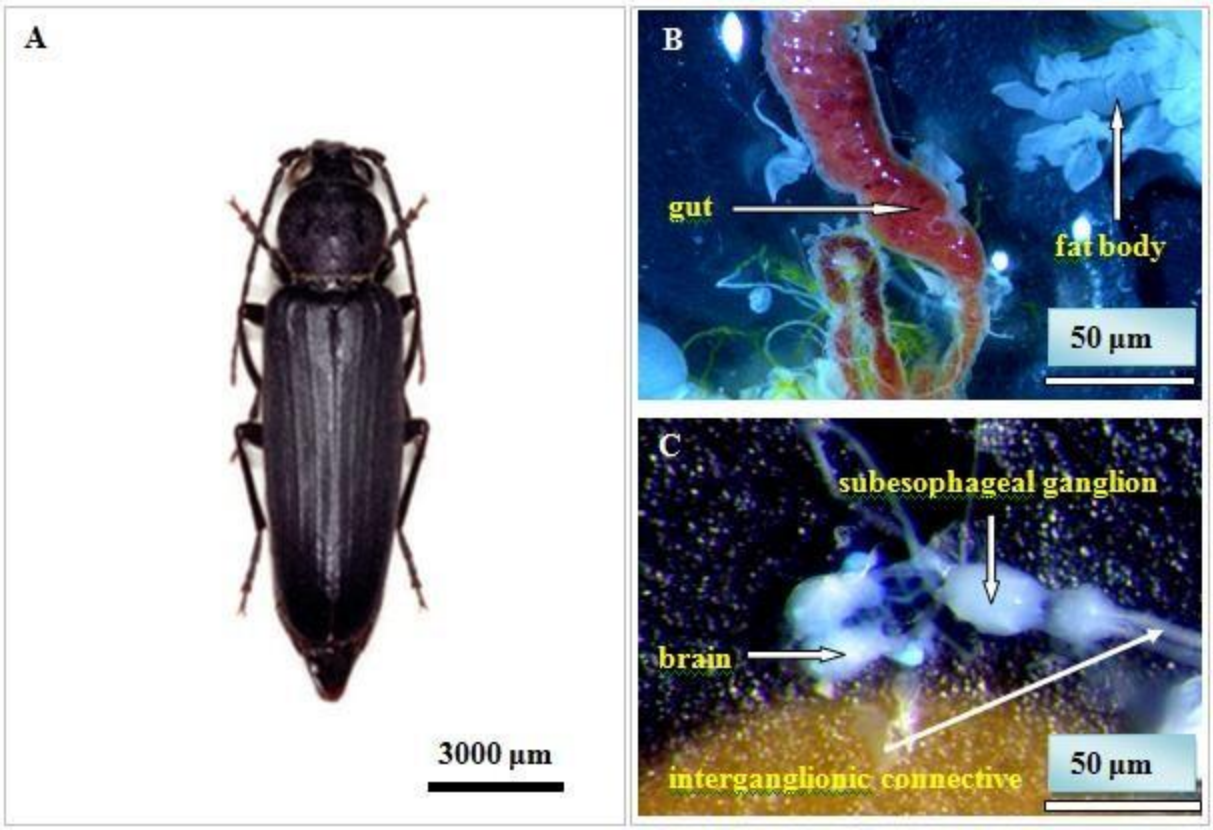

Figure 5.2 *Arhopalus ferus*: Adult (A); a dissected gut and fat body (B); the central nervous system involving the ganglia (C). Images taken using a LEICA M80 microscope, Plant and Food Research Institute, Palmerston North, New Zealand.

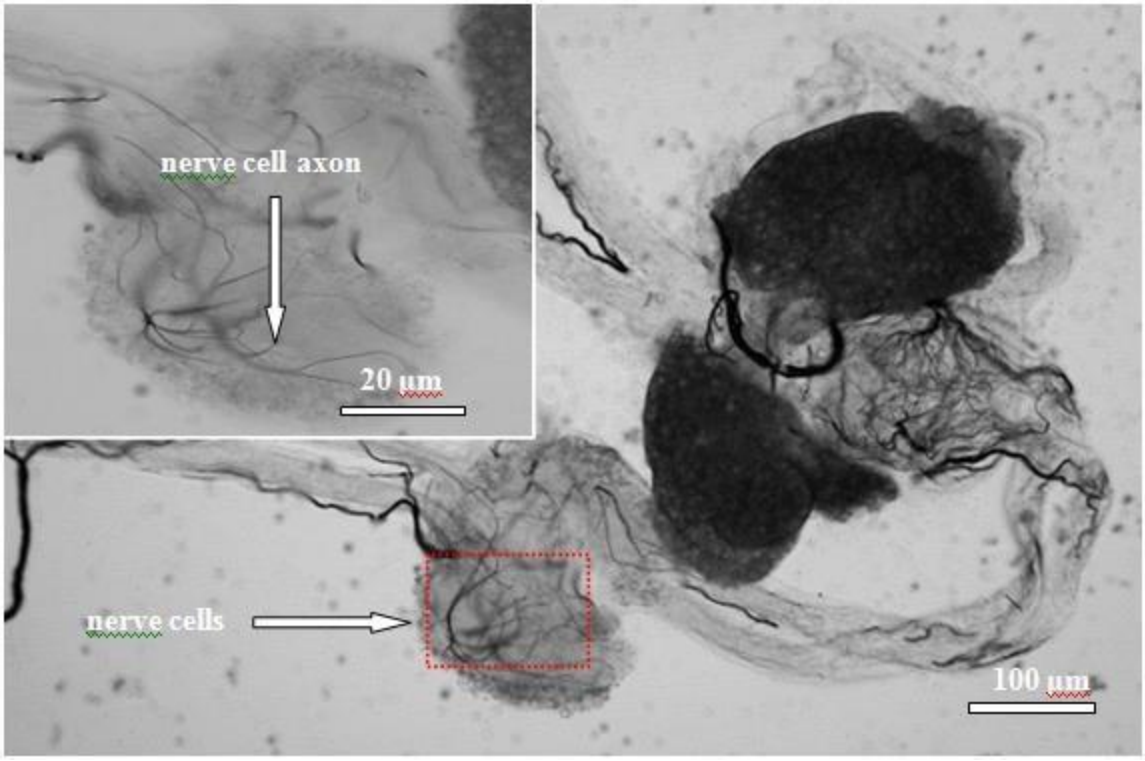

Figure 5.3 Image showing a nerve cell axon (top left) and a section of nerve cells (bottom middle) in the subesophageal ganglion of an adult *Arhopalus ferus*. Images were taken using a LEICA SP5 DM6000B Scanning Confocal microscope, Manawatu Microscopy and Imaging Centre, Massey University, Palmerston North, New Zealand.

5.2.5 Attenuated total reflection fourier-transform infrared (ATR-FTIR) spectroscopy

The attenuated total reflection fourier-transform infrared (ATR-FTIR) (Thermo Fisher Scientific, Nicolet Is5, Madison, USA) spectral analysis was conducted according to Shivanoor and David (2015). This technique provides specific signals that are specific to fumigants and insect tissues. However, the limit of detection is close to the fumigant concentration required for a lethal concentration. Additionally, the technique addresses the problem of getting IR absorption spectra of dry samples that are different from IR spectra of cells in their natural aqueous state. Therefore, the technique enables the study of mid-IR absorption spectra of live and unfixed cells in solution (Hocdé et al. 2001). Furthermore, ATR-FTIR technique enables direct analysis of biological samples (e,g., dissected insect organs) without further preparation, by simply placing them directly on a diamond crystal which has a refractive index higher than that of the sample to be analyzed (Bunaciu et al. 2015; Barth 2007; Lima et al. 2015, Lane & Seo 2012).

In this study, I used a region of the spectrum that is free from interference of IR signals from the insect tissues. I took advantage of the fact that CN^- gives a signal in a clear region (a "window" from approx. 2,000 cm^{-1} to 2,400 cm^{-1}). The ATR-FTIR spectra were recorded at room temperature (25 ± 1 °C). The dissected insect organs were placed individually on the diamond crystal spot of the machine and the pressure tip was lowered and pressed gently, scanned, and the spectra were directly analysed using OMNIC (Omnic 7.1, Thermo Electron Corporation, Madison, WI, USA) software package.

5.2.6 Data processing and analysis

Data collected from the control and CH_3CN-treated whole insects and their organs were drawn as graphs using OMNIC software package (Omnic 7.1, Thermo Electron Corporation, Madison, WI, USA). To compare the responses of CN^- in CNS ganglia of CH_3CN-treated (675270 g/m^3) *A. ferus* and *H. ligniperda* adults, a paired-samples t-test was conducted.

5.3 Results

The results indicate that CN^- was present in the absorbance spectra (CN^- peaks were located ~2,000 cm^{-1} – 2,400 cm^{-1}) of all 20 adult insects (both species) that were exposed to 675270 g/m3 of CH_3CN (Fig. 5.4 shows a sample peak for *H. ligniperda*). However, no CN^- peaks were seen in whole insects that were exposed to 35540.55 g/m3 or 177703 g/m3 and in the control insects that were left untreated.

Fig. 5.5 shows a peak of CN^- present in a CH_3CN-treated (675270 g/m3) whole *A. ferus* adult, compared to no peak in the untreated control. The results also show the presence of CN^- from a sample spectrum for 2 whole adult *H. ligniperda* insects treated with CH_3CN (675270 g/m3) (Fig. 5.6).

For the dissected organs (i.e., CNS ganglia, fat body and gut), CN^- was detected only in the CNS ganglia of CH_3CN-treated *A. ferus* (Figs. 5.7) and *H. ligniperda* (Fig. 5.8). The response for CN^-

in the CNS ganglia of the 20 adult *A. ferus* (2259.877 cm^{-1} ± 1.71) was significantly higher than that of the 20 adult *H. ligniperda* (2256.825 cm^{-1} ± 0.32) (t = 1.72, p = 0.047). For both species, CN^- was absent in CH_3CN-treated and untreated fat bodies and guts.

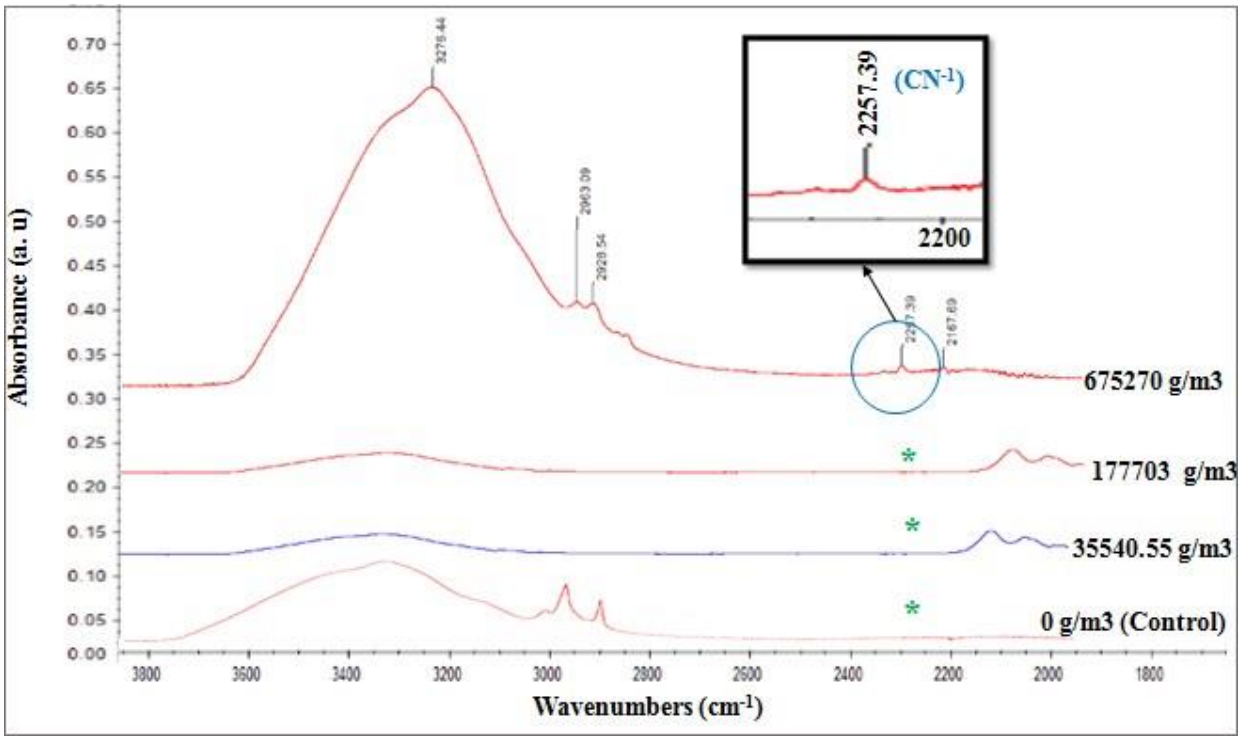

Figure 5.4 Representative ATR-FTIR spectra showing CN^- peak in *Hylurgus ligniperda* adults treated with 675270 g/m3 methyl cyanide-treated. No CN peaks were detected on insects treated with other concentrations of CN.

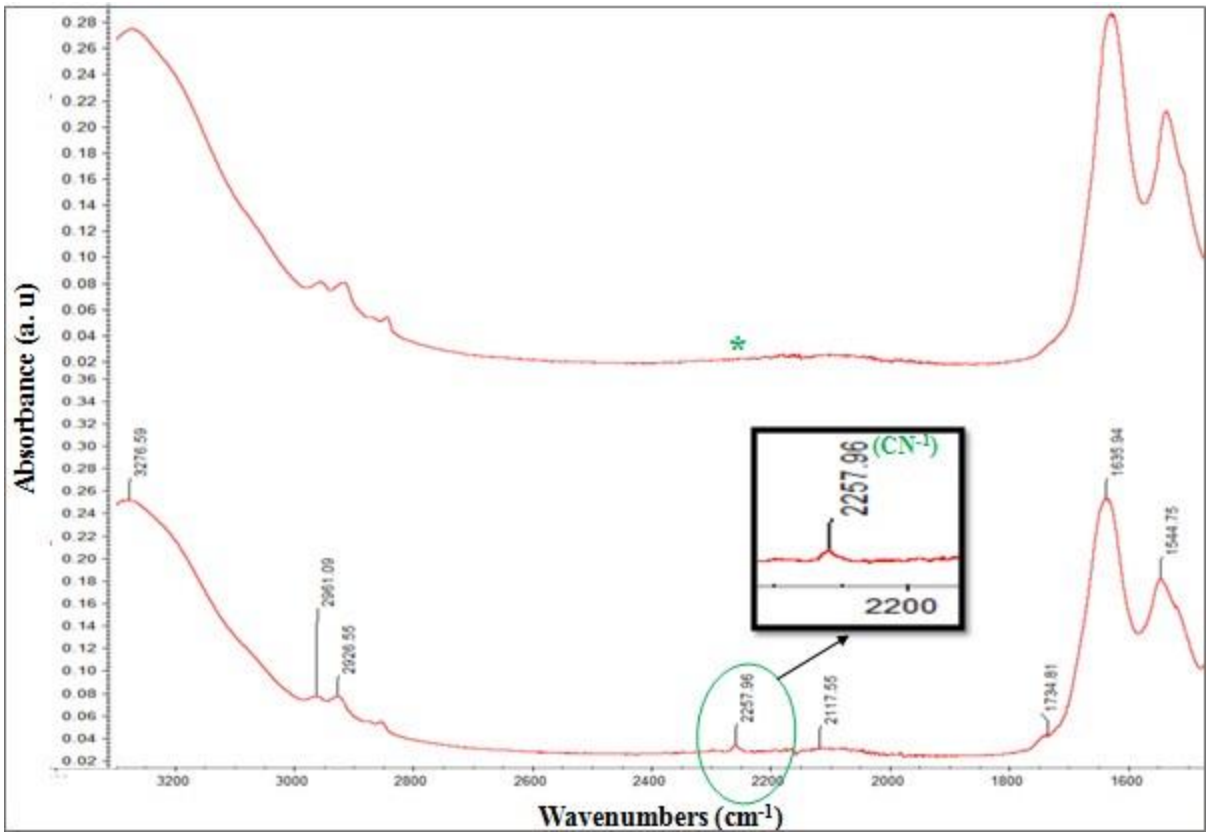

Figure 5.5 Representative ATR-FTIR spectra showing the presence of CN^- in methyl cyanide-treated whole *Arhopalus ferus* adult (lower graph) vs the untreated control with no CN- detected (upper graph).

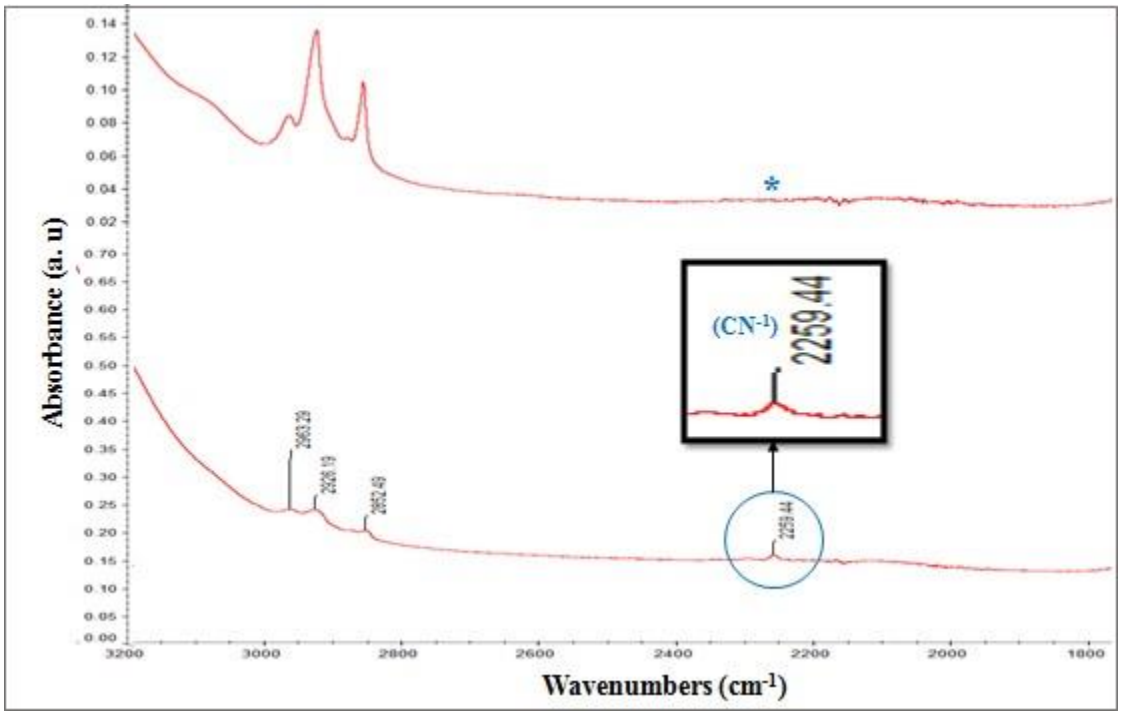

Figure 5.6 Representative ATR-FTIR spectra showing the presence of CN^- in methyl cyanide-treated whole *Hylurgus ligniperda* adult (lower graph) vs the untreated control with no CN^- detected (upper graph).

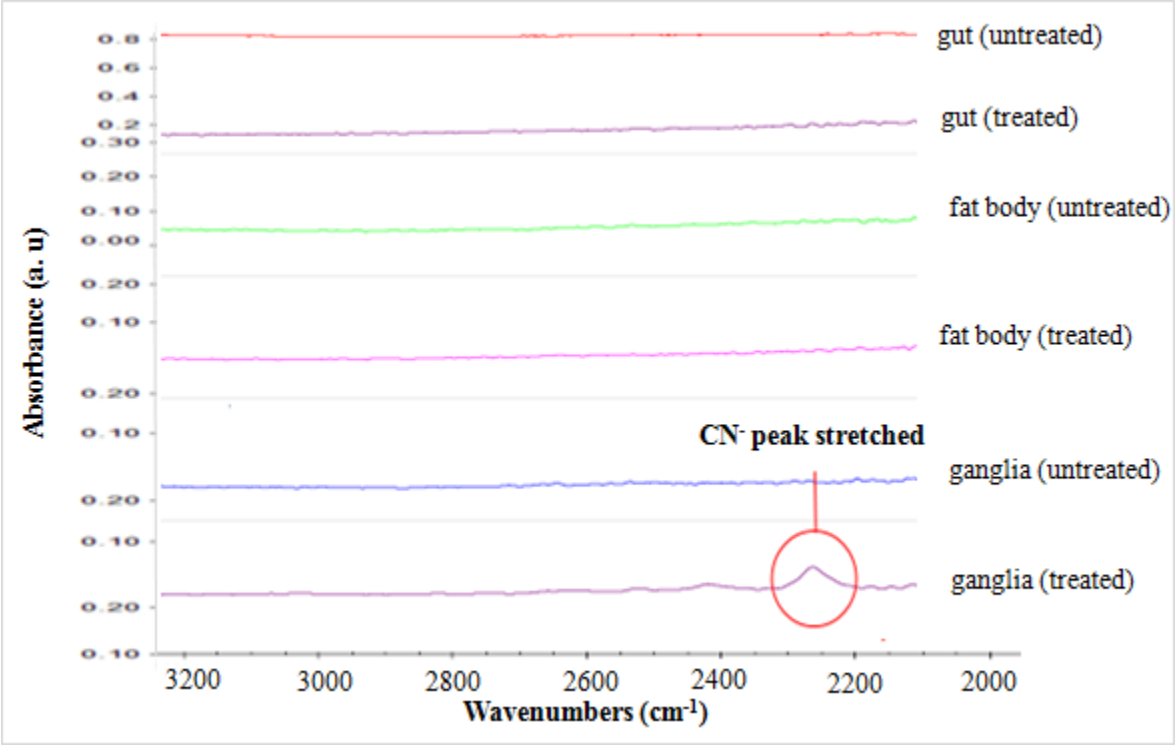

Figure 5.7 Representative ATR-FTIR spectra showing the fate of CN^- in dissected body organs of control (untreated) and methyl cyanide-treated adult *Arhopalus ferus*.

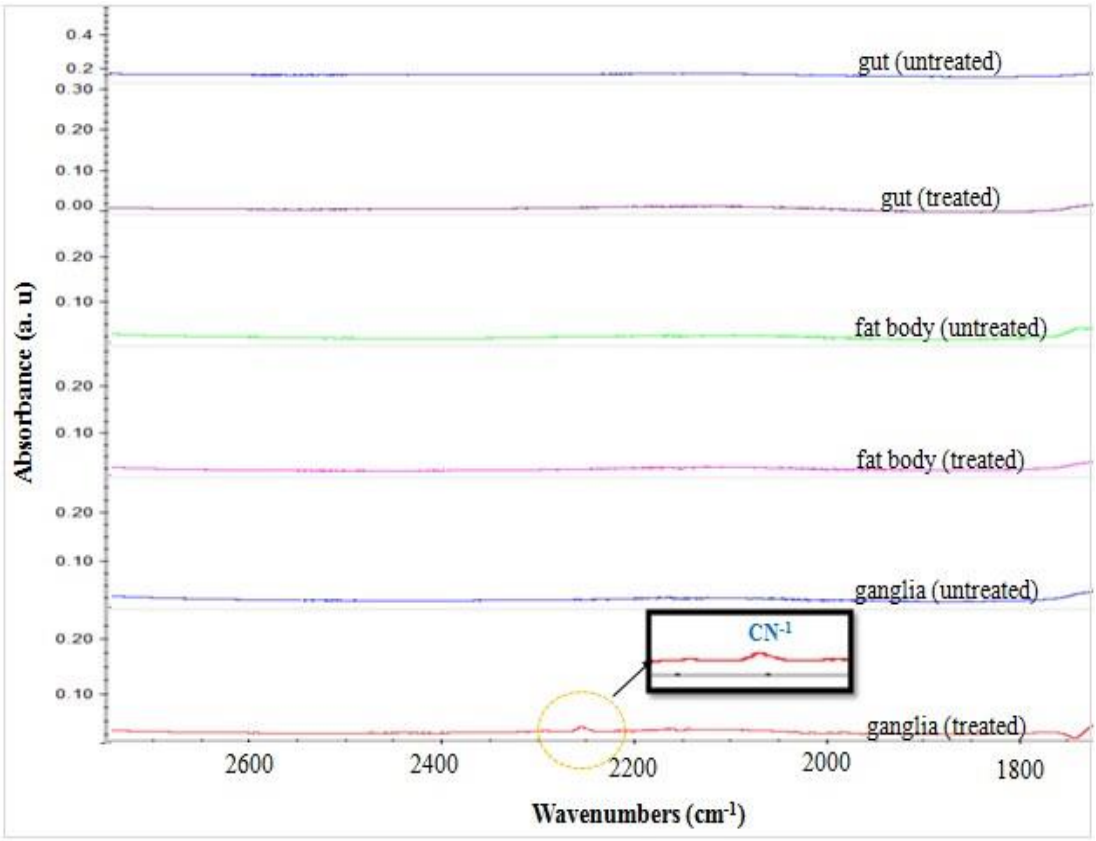

Figure 5.8 Representative ATR-FTIR spectra showing the fate of CN- in dissected body organs of control (untreated) and methyl cyanide-treated adult *Hylurgus ligniperda*.

5.4 Discussion

The present study suggests that the CNS ganglia but not the gut and fat bodies might be a target organ for CN^- in both *A. ferus* and *H. ligniperda* adults because CN^- ion was detected in only the CNS ganglia of CH_3CN-treated *A. ferus* (Figs. 5.7) and *H. ligniperda* adults (Fig. 5.8). These results agree with Isom et al. (1999a), who reported that CN^- is a potential neurotoxin, with direct effects on the CNS, particularly the tissues that have high oxygen needs for metabolic activities such as the supraesophageal ganglion or the brain. Neuronal activities including the restoration of ion transfers by postsynaptic currents, action potentials and neurotransmitter uptake contribute to the brain's energy needs (Ames & Li 1992; Siesjö 1978). Considering that the insect ganglia mainly comprise a network of neurons (Hess 1958; Witthöft 1967), CN^- might have accumulated in the nerve cell axons (Fig. 5.3) and affected synaptic function, but this was not investigated here. The accumulation of toxic substances in the CNS ganglia is known to directly suppress synaptic transmission in some insects (Najar-Rodriguez et al. 2008). Since the efficiency of energy metabolism in the insect CNS is highly dependent on oxygen supply to the nervous tissues, I assume that their high demand for oxygen might make ganglia a prime target for CN^- inhibition. The higher response to CN^- shown in the CNS ganglia of CH_3CN-treated adult *A. ferus* (higher peak values) supports the susceptibility of the species (Najar-Rodriguez et al. 2015a; Pranamornkith et al. 2014d) and the tolerance of *H. ligniperda* to cyanide-containing EDN (Najar-Rodriguez et al. unpublished data).

The absence of CN^- in whole insect bodies after exposure to EDN in the preliminary experiment suggests that the fumigant might not have broken down into HCN/CN^- in insect bodies. This agrees with Hall et al. (2018a) who found that EDN doesn't break down into HCN in treated space. Alternatively, EDN might have been converted to an acyanogenic form that was not detected by the ATR-FTIR technique used, as some insects are capable of modifying cyanogenic compounds by making them acyanogenic (Engler et al. 2000). Therefore, CN^- might not be a break-down product of EDN within insect bodies. No evidence of CN^- in the gut and fat bodies indicates that the insects do not store the ions in these organs. The insect gut is hydrophilic and might have eventually converted CN^- to a less detectable form (e.g., SCN) for the ATR-FTIR used. The action of detoxifying enzymes in the gut might also be a possible reason why CN^- was not detected in there. Furthermore, the absence of CN^- in the fat bodies might be due to their

hydrophobic nature, making it non-reactive to the CN^- ion, which is hydrophilic. In a related case, the larvae of *P. rapae* detoxified CN^- to less harmful *β*-cyanoalanine and thiocyanate in their gut (van Ohlen et al. 2016).

Insects and some arthropods are capable of adopting very efficient means of detoxifying CN^- in their bodies (Duffey & Blum 1977; Meyers & Ahmad 1991; Witthohn & Naumann 1987; Wybouw et al. 2014) by activities of rhodanese (Beesley et al. 1985; Cerletti 1986) and/or *β*-cyanoalanine synthase (Meyers & Ahmad 1991; Miller & Conn 1980) enzymes. The target organs and patterns of distribution of such detoxifying enzymes within body tissues may differ across species. The activities of the enzymes in a particular tissue may reflect the ability of that tissue to detoxify CN^-. Further studies aiming to measure the activities of detoxifying enzymes in EDN-treated vs untreated insects could provide more insights into the roles of these enzymes in insects' tolerance or susceptibility to EDN.

In conclusion, the current study has identified CNS ganglia of both *A. ferus* and *H. ligniperda* adults as a possible prime target for CN^-. Cyanide was, however, absent in the gut and fat bodies of both species. Considering that exposure to CN^- was through inhalation, the current results suggest the possibility of two pathways in both species: (i) some CN^- might have passed through the tracheal system into the CNS ganglia, resulting in the death of insects and (ii) some CN^- might have also passed through the tracheal system and combined with elements such as sugars (Bond 1961a) within the insect bodies and be converted to other compounds that might not have been detected by the ATR-FTIR. Therefore, the relative amounts of CN^- that passed through any of the pathways might have accounted for the toxicity differences between the three different concentrations of methyl cyanide used here. Also, adult *A. ferus* accumulated more CN^- compared to *H. ligniperda*. It is assumed that factors that inhibit the cellular or target site access of CN^- in *H. ligniperda* may likely be reasons why this species is more tolerant to EDN than *A. ferus*.

CHAPTER 6 General Discussion and Conclusions

6.1 Introduction

New Zealand exports *P. radiata* timber and log products worth approximately NZ$ 5 billion (Armstrong et al. 2014; MPI 2020), annually. The primary sector, which includes forestry, constitutes 7 % of the Gross Domestic Product of the country (MPI 2020). After dairy and meat, wood products are New Zealand's third-largest primary industries export earner. Since the sustainability of this trade is a key goal for the New Zealand forestry industry, the export markets require effective control of quarantine insects that accompany wood products. Fumigation is the most economically practical way to control insects and other pests at the postharvest level (Fields et al. 2004). Currently, MB fumigation is the most effective approach to quarantine and pre-shipment treatments for the international markets. However, because MB is ozone-depleting, its use in New Zealand henceforth will require recapture or destruction to control atmospheric emissions (Hall et al. 2017). Therefore, alternatives are being searched.

Ethanedinitrile has shown great promise as a good alternative (Pranamornkith et al. 2014c). However, EDN toxicity varies across insect pests (Najar-Rodriguez et al. 2015b; Pranamornkith et al. 2014c; Hooper et al. 2003), implying that the mode of action might differ among species. Therefore, investigative research into the fumigant mode of action against insects is required. The overall goal of the present study was to understand mechanisms behind the toxicity of EDN to insects. In this chapter, I summarised and discussed the current knowledge relevant to my study, main findings and drew conclusive remarks of the thesis.

6.2 Literature Review

6.2.1 Key forest insects

In Chapter 2, I first reviewed literature regarding the knowledge on two key forest beetles of quarantine interest to New Zealand's trading partners: *H. ligniperda* and *A. ferus*. Both beetles originate from Eurasia, but have widely spread across Europe and other parts of the world through international trade of wood products (Aukema et al. 2010; Brockerhoff et al. 2006a; Wang 2017). In New Zealand, *P. radiata* is the main host for both beetles. Their presence on

logs harvested for export poses biosecurity risks, for which reason importing countries insist on strict implementation of phytosanitary measures.

6.2.2 Ethanedinitrile (EDN) fumigant

Ethanedinitrile is a new fumigant with a great potential as a MB alternative in the quarantine treatment of timber and wood products. Joseph Louis Gay-Lussac first produced a small amount of EDN in 1815, using thermal decomposition of silver cyanide (Fierce & Millikan 1958). However, the fumigant potential of EDN was first discovered by CSIRO in 1996 (Waterford et al. 2008). EDN has a threshold limit value of 10 ppm (v/v), which compares favourably with MB (5 ppm) and phosphine (0.3 ppm) (Ren et al. 2006). EDN has an excellent spectrum of efficacy against soil, insect pests, nematodes, weeds, fungi and diseases (Ducom 2006). In 2011, the fumigant was registered in Australia as a disinfestation treatment for logs and sawn timber (Pranamornkith et al. 2014d), after which it became available for research purposes in New Zealand (Najar-Rodriguez et al. 2015a). Recently, it was approved as a treatment option for the control of adult *A. ferus* insects on Australia-bound logs and wood products from New Zealand (MPI 2018). Currently, EDN is not yet registered in New Zealand and cannot be used at a commercial level. However, an application has been submitted to the New Zealand E.P.A. for permission regarding its application in New Zealand (APP202804).

6.2.2.1 The efficacy of EDN against insect pests

The potential of EDN as an effective fumigant against a wide range of insects has been documented. The first reference to studies regarding the fumigant efficacy on wood-related insects was made by CSIRO (1996). Even though EDN was effective at controlling three termite species, only 10-15 termite workers were used in this research. Wright (2000) also conducted the above experiments and provided the same results, but gave no sufficient evidence to prove that EDN had potential use against the insects. EDN was effective against *A. glabripennis* (Ren et al. 2003), but there was no mention of the initial concentration used and the number of replications in their article.

In another research, EDN was generally excellent at controlling a range of stored-product insects, except immature stages of *Sitophilus* sp. (Hooper et al. 2003). EDN has also shown great

potential for the control of insect-infested logs (Cho et al. 2011). More recent studies show that all life stages of *A. ferus* were susceptible to the fumigant (Najar-Rodriguez et al. 2015a; Pranamornkith et al. 2014c), but adults of *H. ligniperda* were highly tolerant (Najar-Rodriguez et al. unpublished data). Najar-Rodriguez et al. (2015a) also found that EDN was more toxic to the adult life stages of *A. ferus* than MB. Ramadan et al. (2020) also reported that EDN was toxic to all life stages of *L. Serricorne* and *R. dominica*, but toxicity levels varied depending on insect species, strains and life stages. These results suggest that the mode of action might be species-specific or life stage-specific and more complex than previously thought. Therefore, investigations into its mode of action against insects were needed to explain the observed differences.

6.3 Effect of insect anatomy on tolerance to EDN

Basic information on the presence and functions of adaptations of insects to xenobiotic conditions is critical in understanding how pesticides (including fumigants) kill insects and how such ideas may be used in designing effective control strategies. Chapter 3 of this thesis (i) investigated potential differences in EDN tolerance between adult *A. ferus* and *H. ligniperda* by examining their cuticle and spiracle anatomies and (ii) identified the main route (s) of entry for the fumigant into both insects.

6.3.1 Measurement of insect cuticle thickness and spiracle area

I examined the anatomy of the cuticle and spiracles of *A. ferus* and *H. ligniperda* adults to explain differences in tolerance to EDN between the two forest pests. I found that the cuticle of *H. ligniperda* had more chitin layers than that of *A. ferus*, suggesting the possibility of delayed penetration of EDN through that route in the former species. Cuticle thickness varied greatly in different body parts of both beetle species, implying that cuticle thickness may not account for the tolerance differences between both species, and rather, cuticle composition matters.

Results of a comparative study on spiracle structure, position, area and functions showed that both *A. ferus* and *H. ligniperda* possessed 9 pairs of visible spiracles: two thoracic and seven abdominal, as reported in other coleopterans (Ritcher 1969). *A. ferus* had larger total spiracle area than *H. ligniperda*, suggesting the possibility of inhaling more EDN (Gidiglo et al. 2018).

As suggested by Lighton et al. (1993) for insects, the rate of exchange of gases (including EDN) depends on the area of spiracle openings; larger spiracle openings may offer more entry and exit pathways. In line with Chown et al. (2007a), comparison of the tracheal investment and body size of both insects suggests that *A. ferus*, as the larger insect, may have lower mass-specific metabolic and gas exchange rates, which may increase EDN efficacy. Because individual spiracle sizes and their external and internal closing mechanisms varied between and within species, the contributions of each spiracle in the exhalation or inhalation of EDN might differ. The efficacy of some insecticide classes on insects has been associated with the closeness of spiracles to the insect brain (Sugiura et al. 2008). Huber (1965) observed that the mesothoracic spiracles could offer a rapid entry of gases into the insect bodies and provide the quickest route to the CNS. Therefore, the location and morphological features of these spiracles were also examined. The results of this thesis have shown that of all spiracles on both species, mesothoracic spiracles were closest to the brain (Chapter 3, Fig. 3.4).

The results of further morphological studies conducted on the mesothoracic spiracles have also shown that the size, number, shape, height, proximity and orientation of setae covering the openings were different in both beetle species. The spiracular setae in *A. ferus* were large, branched or bi-pectinate, fewer and spacious, but those in *H. ligniperda* were straight, simple acuminate, small, short and more compact. Sukontason et al. (2006) found similar setae in the mesothoracic spiracles of *M. domestica* and reported that their presence could prevent the entry of dust. Chapman and Chapman (1998) added that, even though the mesothoracic setae appeared in different sizes and orientation, their main function was filtering dust and other small particles. The shorter, more compact and denser setae around the mesothoracic spiracles of *H. ligniperda* may trap humid air (Nagpal et al. 2003) and create humid conditions for EDN to quickly combine with moisture (Emekci 2010) around the spiracular openings. Even though the reaction may not result in the break-down of EDN to HCN and other products, the break-down products may fail to successfully pass through the tracheal system to the final target site(s). Furthermore, the presence and contribution of such special setae in maintaining humid conditions around the openings of the mesothoracic spiracle of *H. ligniperda*, possibly causing intermittent closure of the spiracles, may limit the uptake of EDN into the insects. Based on the observed morphological features, *H. ligniperda* may be more efficient in preventing entry of EDN through the mesothoracic spiracles, compared to *A. ferus*. Given that the inner closing lids in the

metathoracic spiracles of *A. ferus* showed large narrow space inside the atria, but that of *H. ligniperda* comprised both inner and outer lids and valves with smaller size, with less space in the atria, more effective regulatory control of EDN entry into *H. ligniperda* is expected.

In addition, adult *A. ferus* were significantly larger than *H. ligniperda*. Considering that the rate of exchange of substances largely depends on the surface-area-to-volume ratio of insects (Kühsel et al. 2017), the large surface area of adult *A. ferus* suggests that the insect possesses small surface-area-to-volume ratio. Large surface area means more exposure to the environment and the vulnerability of body cells to EDN exposure. Also, large body surface area may lead to uptake of more EDN, due to the increased number and size of cells. The results validate the hypothesis that differences in the location, structure and function of spiracles on *A. ferus* and *H. ligniperda* adults contribute to the observed differences in EDN tolerance between these two species.

6.3.2 Ethanedinitrile route (s) of entry into target insects

The mortality of fumigated *H. ligniperda* with all thoracic spiracles blocked was not statistically different from the unblocked controls. However, for *A. ferus*, the results suggest that the thoracic spiracles are primary entry routes for EDN into the insect, as blocking these spiracles led to lower mortalities in the fumigated beetles (Table 3.4). Similarly, the rate of uptake of vaporized pyrethroid into *M. domestica* was decreased due to the blocking of the mesothoracic spiracle (Sumita et al. 2016). Air that enters the insect body through the mesothoracic spiracles is first channelled to the brain and then to the rest of the CNS (Burrows 1980), and the thoracic spiracles are closest to the brain, which forms part of the CNS (Sugiura et al. 2008). The abdominal spiracles, however, might be the primary exit routes of the fumigant. Further work is required to relate EDN uptake to spiracular condition, to provide detailed explanations for spiracular response. Since HCN is known to cause complete inhibition of muscle contractibility (Bond 1961a), relaxation and contraction of spiracular muscles are expected during fumigation. Therefore, assessment of spiracular conditions during fumigation may give detailed explanations regarding the patterns of spiracular intake of EDN by *A. ferus* and *H. ligniperda*. Additionally, the significantly higher mortality of fumigated insects from both species with blocked abdominal spiracles, and lower mortality of insects with blocked mesothoracic spiracles suggest that

abdominal spiracles might be important in the process of exhalation of the fumigant from the insect body.

The overall results from the spiracle-blocking experiments suggest that (i) spiracle-blocking did not prevent the entry of EDN into the insects and (ii) EDN might have penetrated the insects through other body parts. The penetration of substances into insects through other body parts, despite blocking spiracles, has been observed in some species. For example, after blocking spiracles of the *H. ligurriens* larvae, oxygen still penetrated the insects (Fraenkel & Herford 1938). Also, a toxic quantity of MB had accumulated in the larvae of *T. mauritanicus* beetles, despite blocking access to tracheal entry of the fumigant into the insects (Monro 1959).

The inability of spiracle-blocked *A. ferus* and *H. ligniperda* insects to recover after EDN fumigation might be due to the fact that blocking prevented the escape of the fumigant and enabled close contact and reaction between the fumigant molecules and moisture in the body cells (Emekci 2010), resulting in insect death. In a related research by Bond (1961a), the rate of entry of HCN, a decomposition product of EDN into *S. granarius* depended on the rate of fixation of the fumigant in the insect tissues. Therefore, it may be right to conclude that conditions that bring fumigant molecules closer to the internal body cells may enable easier diffusion and fumigant efficacy. Furthermore, the gradual recovery of unblocked and fumigated insects (from both species) may suggest their ability to reduce the toxic effect of EDN (by exhalation) through the unblocked spiracles.

Cuticle-coating with nail polish significantly decreased insect mortality due to fumigant (Table 3.2), suggesting a reduction in its entry due to coating. Comparing the higher mortality rates when blocking all thoracic spiracles with mortality rates when coating the cuticle, the results suggest that the tracheal system plays a minor role in the overall uptake of EDN into adult *H. ligniperda*. Rather, the cuticle may be regarded as the primary route. The insect cuticle has been cited as the main route of entry for different types of insecticides into insects (Galley 1967; Sumita et al. 2016; Najar-Rodriguez et al. 2008). For instance, contact insecticides are known to first enter insect bodies through the cuticle and proceed to the CNS, using the haemolymph as a medium (Grissom Jr et al. 1989; Matsumura 1963; Yu 2008). Given that (i) the cuticle of *H.*

ligniperda possessed more chitin layers and denser than *A. ferus* (Fig. 3.10), (ii) the cuticle is regarded as the main route of entry into the insect, and (iii) the total spiracle area of *H. ligniperda* was comparatively smaller than that of *A. ferus* and could offer resistance to the uptake of EDN, delayed penetration of the fumigant into *H. ligniperda* might be an important factor for the observed tolerance of this species.

In conclusion, my findings strongly suggest that the possession of smaller spiracle areas, larger body surface area and more chitin layers in the cuticle could be morphological adaptations that led to adult *H. ligniperda* being more tolerant to EDN than *A. ferus*. Furthermore, the results have revealed that the cuticle might be the main route of entry for EDN into the body of adult *H. ligniperda*, whilst the thoracic spiracles played a minor role. For adult *A. ferus*, thoracic spiracles may be regarded as the main route of entry for EDN. The outcome of these findings contributes to the general understanding of (i) the various mechanisms behind the toxicity of EDN and (ii) the mode of entry of the fumigant for the effective control of insects.

6.4 Effect of EDN fumigation on insect behaviour

Insects are capable of withstanding insecticide exposure through various physiological adaptations or behavioural modifications, by avoiding or minimizing their encounter with the compounds, but very few studies have investigated behavioural effects of insecticides and their significance in better understanding their modes of action. Despite the potential of EDN as an alternative fumigant to MB for control of forest insects, no research had paid attention to its effects on insect behaviour. Therefore, in Chapter 4 of this study, I evaluated the behavioural responses of adult *A. ferus* and *H. ligniperda* to EDN. Exposure of both species to EDN resulted in decreased walking activities, with *H. ligniperda* showing greater and more consistent reductions in walking speed, walking distance and exploration rate. These reductions occurred at different times, according to concentrations and periods of exposure. In *A. ferus*, EDN fumigation led to an initial excitation, followed by instant paralysis and immobility. After fumigation, a different response between the two species was observed with *H. ligniperda* exhibiting slower recovery and *A. ferus* no recovery in most cases. It may be suggested that the ability of *H. ligniperda* to recover after fumigation could be part of a mechanism that allows this species to withstand exposure to higher concentrations of EDN than *A. ferus.*

Such reduced walking activity as a protective behaviour may be as a result of decreased metabolism and respiration rates in the insects, caused by the fumigant (this is currently unknown) thereby minimizing its uptake. Reduction in walking activity, as a means to minimize and withstand insecticide exposure has been reported in some insects (Wang et al. 2000; Cox et al. 1997; Watson et al. 1997; Guedes et al. 2008; Pimentel et al. 2007, Pimentel et al. 2008). Guedes et al. (2011) reported that exposure to phosphine fumigant reduced walking activity of *S. zeamais* and that condition was likely to minimize respiration rate of the insects and result in reduced uptake of the fumigant. The current results have established an association between EDN tolerance and lower walking activity in the tolerant species (*H. ligniperda*), which potentially minimized the toxic effect of the fumigant against it. The EDN mode of action against adult *A. ferus* seems to be through temporal stimulation of walking activity, followed by paralysis and death. That against adult *H. ligniperda* has been seen to involve narcosis, which occurred at different times according to concentrations and time of exposure to EDN. During EDN fumigation, adult *H. ligniperda* first went through paralysis, followed by a reduction in walking activities; a situation which might have led to reduced respiration rates as a protective effect to enable them to tolerate higher than normal doses of the fumigant. Though some level of narcosis had occurred in both species, it was more severe in *H. ligniperda*. This behavioural research has improved my understanding of EDN mode of action and could offer industry the opportunity to optimize treatment parameters.

In conclusion, exposure of both species to all EDN concentrations led to decreased walking activities. Such reduced walking activity is likely to be linked to lower respiration rate of the insects, thereby minimising their uptake of EDN. During fumigation, EDN exposure at higher concentrations induced more pronounced narcotic responses in *H. ligniperda* (similar to "knock-down", with insects falling off several times in the arena). In *A. ferus*, EDN fumigation initially caused hyperactivity in the insects, followed by instant paralysis and reduction in walking activities. In *H. ligniperda*, the fumigant first caused instant paralysis, followed by a reduction in walking activities. After the fumigations, *H. ligniperda* exhibited gradual recovery, suggesting the occurrence of partial respiratory inhibition and the possibility of narcosis being used as a protective mechanism by this species. This could be part of a mechanism that allows *H. ligniperda* to withstand exposure to higher concentrations of EDN better than *A. ferus.*

6.5 Evaluation of Cyanide toxicity in selected Organs of adult *A. ferus* and *H. ligniperda*

Given that the toxicity of EDN may be determined by the action of CN^-, the fate of this ion within insect bodies may provide clues regarding its mode of action. Based on the hypothesis that the toxic action of CN^- against insects might relate to its accumulation in insect organs, Chapter 5 investigated the possibility of CN^- accumulation in the gut, fat bodies and ganglia of adult *A. ferus* and *H. ligniperda*, using ATR - FTIR technique. I first conducted preliminary tests on whole target insects with EDN using the technique but detected no traces of CN^- in the insect bodies. Therefore, I decided to use methyl cyanide (CH_3CN) as a surrogate compound to EDN instead. The absence of CN^- in whole insect bodies after exposure to methyl cyanide suggests CN^- might not be a break-down product of EDN within insect bodies.

The study involving CH_3CN has identified CNS ganglia of two insect species as a prime target for CN^-. The results agree with Isom et al. (1999b), who reported that CN^- as a potential neurotoxin has a direct effect on the CNS, particularly the tissues with high oxygen needs for metabolic activities such as the supraesophageal ganglion or the brain. Some of the activities that contribute to the brain's energy needs are restoration of ion transfers by postsynaptic currents, action potentials and neurotransmitter uptake (Siesjö 1978; Ames & Li 1992). Considering that the insect ganglia mainly contain a network of neurons (Hess 1958; Witthöft 1967), CN^- might have accumulated in the nerve cell axons (Fig. 5.3), and affected synaptic function. The accumulation of toxic substances in the CNS ganglia directly suppresses synaptic transmission in some insects (Najar-Rodriguez et al. 2008). Since the efficiency of energy metabolism in the insect CNS depends greatly on oxygen supply from the nervous tissues, it is assumed that their high demand for oxygen might make ganglia a prime target for CN^- inhibition.

The fact that CN^- was not detected in the gut and fat bodies suggests that the ion may not have accumulated in the organs. Given that the insect gut is hydrophilic in nature and has high affinity for moisture, CN^- might have eventually been converted by enzymes to another form (e.g. SCN) (van Ohlen et al. 2016), become less detectable by the ATR-FTIR used. In the gut of *P. rapae* larvae, CN^- was detoxified to less harmful β-cyanoalanine and thiocyanate (van Ohlen et al. 2016), suggesting that the absence of CN^- in the gut might be due to the action of detoxifying enzymes. In addition, the hydrophobic nature of fat bodies might have made them non-reactive

to the CN^- ion, which is rather hydrophilic, hence, the absence of CN^-. The higher amount of CN^- in the CNS ganglia of CH_3CN-treated adult *A. ferus* (higher peak values) supports the reported susceptibility of the species to EDN (Najar-Rodriguez et al. 2015a; Pranamornkith et al. 2014c). Also, the tolerance of *H. ligniperda* to cyanide-containing EDN was shown by the appearance of lower CN^- peak values (Najar-Rodriguez et al. unpublished).

Given that in this experiment CN^- exposure was through inhalation, the results suggest the possibility of two pathways that could explain the observed differences in toxicity: (i) some CN^- might have passed through the tracheal system into the ganglia, resulting in the death of insects, and (ii) some CN^- might have also passed through the tracheal system and combined with elements such as sugars (Bond 1961a) within the insect bodies and got converted to other compounds that might not have been detected by the ATR-FTIR.

6.6 General conclusions

Overall, the series of studies undertaken in this thesis have revealed several possible mechanisms behind the toxicity of EDN to adult *A. ferus* and *H. ligniperda*. I also found evidence to support differences in the tolerance of these target insects to EDN. Briefly, the studies have led to the following conclusions:

- Adult *H. ligniperda* possessed morphological advantages that might enable it to tolerate high doses of EDN, compared to *A. ferus*.

- The cuticle of *H. ligniperda* was the main route of entry for EDN. For *A. ferus*, thoracic spiracles were the main route of entry for the fumigant.

- EDN fumigation reduced the walking activity of both species, with greater and quicker reductions measured in adult *H. ligniperda*. This might be a behavioural adaptation that could lead to reduction in fumigant efficacy, thereby minimizing its uptake and the toxic effect of the fumigant in this species.

- EDN mode of action against adult *A. ferus* was through narcosis, followed by paralysis and death. For adult *H. ligniperda*, the killing effect also involved narcosis, which occurred at different times, according to concentrations and time. Though some level of narcosis had occurred in both species, more had been seen in *H. ligniperda*. It may be concluded that during fumigation, adult *H. ligniperda* first underwent paralysis, followed by a reduction in walking activities, probably leading to lowered respiration rates as a protective effect to enable them to tolerate higher doses of the fumigant.

- Central nervous system ganglia are a prime target for CN^- in both species. Additionally, higher response to CN^- shown in the CNS ganglia of CH_3CN-treated adult *A. ferus* (higher peak values) supports the susceptibility of the species and the tolerance of *H. ligniperda* (exhibited lower response) to cyanide-containing EDN.

6.7 Recommendations for future work

The experiments in this thesis addressed mechanisms behind the toxicity of EDN by focusing on just two insects that have demonstrated different levels of tolerance to the fumigant. It would be of interest to know the fumigant effect on other insect species from the same or different orders (including *Sitophilus* sp.). This will help our general assessment and understanding of the fumigant mode of action across different species. Since the results of this thesis have provided information regarding EDN routes of entry into target insects, ideas relevant to how the fumigant toxicity could be enhanced by improving the permeability of the fumigant through the insect cuticle and the spiracles are encouraged.

Even though the current study has identified CNS ganglia as a potential prime target for CN^- in both species, there is still the need to further investigate factors that inhibit the cellular access to CN^- and determine the physiological fate of EDN and its toxicity potential within the insect bodies via detection and monitoring of activities and intracellular distribution of possible CN^- detoxifying enzymes in different tissues (e.g., gut, fat bodies, haemolymph and cuticle). Knowing that thickness of cuticle layers and their compositions differ across and within species, investigations on the cuticular properties and components of *H. ligniperda* in particular are also required because of predictions on the possibility of delayed penetration of EDN into the insects.

Also, this thesis determined spiracle sizes of target insects. Thus, it would be worth measuring the rate of respiration and volume of EDN inhaled and exhaled vs the toxic effect. This will establish a relationship between how much of the fumigant enters and leaves the insects through the spiracles and the rate at which that happens.

The findings of this thesis have confirmed that EDN is effective at controlling adult *A. ferus* (Najar-Rodriguez et al. 2015a; Pranamornkith et al. 2014d), while toxicity against adult *H. ligniperda* remains poor and improvements need to be made to enable the widespread use of this alternative fumigant. My research illustrates that the high tolerance of *H. ligniperda* is likely due to the following features: (i) possession of additional cuticle layers, which may reduce EDN penetration through the cuticle; (ii) possession of smaller body size that may create higher gas exchange between the environment and body cells, enabling EDN molecules to quickly escape from the insect body, and (iii) ability to avoid the fumigant exposure by quickly recognizing its presence and instantly reducing walking activities, which may lead to lowered respiration rates and reduced EDN intake. Therefore, EDN efficacy against adult *H. ligniperda* could be enhanced by improving the fumigant penetration ability and increasing movement of the insect.

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Appendices

Appendix 1

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

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