Factors affecting the disinfestation of pine logs during transit

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Abstract

The effective disinfestation of wood products for trade is critical to halting the spread of global invasive insect species. Invasive insects are being transported inadvertently with wood products, despite increasing awareness of this issue. With the arguably most useful and effective disinfestation tool for wood products, methyl bromide (MB), due to be phased out globally, the issue of finding equally effective replacements is pressing.

Phosphine (PH_3) has been in use for several decades to disinfest unprocessed radiata pine (*Pinus radiata* D.Don) logs during shipment as an alternative to MB in New Zealand. The PH_3 treatment used for pine logs is based on a fumigation schedule for grains. However, little is known about the physiology of the target pests, bark beetles, and how their physiology may affect disinfestation in an enclosed space during log transit. Further, the unique abiotic and biotic variables associated with the fumigation of whole pine logs within relatively airtight ship holds is largely unknown.

In this thesis I focus on three fundamental knowledge gaps related to the use of PH_3 for the disinfestation of pine logs in transit. Firstly, I quantified the respiration of recently harvested radiata pine logs during storage in an enclosed space. I showed how respiring pine logs dramatically changed the surrounding atmosphere to severely hypoxic (low O_2) and hypercapnic (high CO_2) within days of containment. I also demonstrated that there were strong seasonal differences in the amount and rate of CO_2/O_2 from the log sections.

Secondly, I determined the respiratory tolerances and gas exchange patterns of the bark beetle *Hylurgus ligniperda* (F.), a target pest species in radiata pine disinfestation. Building on the atmospheric data I obtained from the respiration of radiata pine logs, I tested the respiratory response of *H. ligniperda* to varying levels of O_2. *H. ligniperda* exhibited a continuous ventilatory pattern and was highly tolerant of hypoxic atmospheres across all three life stages tested: larvae, pupae, and adults. Pupae were the most tolerant to hypoxic atmospheres.

Thirdly, I analysed the efficacy of PH_3 against all life stages of *H. ligniperda*. Overall, 100% efficacy was not achieved for all life stages; pupae were the most tolerant of exposure to PH_3. Contrary to other studies, I found the egg life stage was the most sensitive to PH_3. PH_3 efficacy was most improved by a longer exposure, rather than an increase dose.

Measuring the respiration of both pine logs and bark beetles has highlighted the challenge of controlling endophagous insect pests in an enclosed space. Overall, my research suggests that control treatments could be less effective against bark beetle pests due to their ability to regulate their respiration under the severely hypoxic atmospheres that develop during the transportation of whole pine logs. This adaptation may reduce the insect's uptake of PH_3 through the respiratory system and, hence, may explain why there has been varied operational success with the current treatment schedule.

Dedication

I dedicate this thesis to my parents, Robina and Tony Devitt

Thank you for all your love and support

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This is going to be long…

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Co-Authorship Form

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Nature of contribution by PhD candidate	Conceptual development of the study design. Carried out data collection, analysed the data, wrote the paper
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Matthew KD Hall	Conceptual development of the study. Contributed to editing the paper.
Adriana J Najar-Rodriguez	Conceptual development of the study. Contributed to editing the paper.
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Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate's contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

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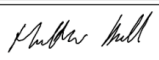
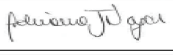
Matthew KD Hall		17 September 2020
Adriana J Najar-Rodriguez		17 September 2020
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Chapter 1 – General introduction

1.1 Biological invasions and biosecurity

1.1.1 Overview

It is estimated that over 500,000 species of exotic plants, animals and microbes have been either intentionally or unintentionally introduced to novel areas worldwide (Pimentel et al., 2008). Although the majority of these introductions are thought to have negligible impacts on their new environments (Ricciardi et al., 2013) there is a relatively small subset that can become detrimental when introduced to a new environment (Elton, 1958). Ecosystem change, native species declines and extinctions are associated with invasive species (Clavero & García-Berthou, 2005; Pyšek et al., 2020). There are several factors associated with this process that establish a species as invasive, such as a lack of natural enemies in the new environment (e.g. enemy escape hypothesis; Keane & Crawley, 2002). A lack of defences to the new species by the native environment/organisms (i.e. evolutionary naïve; Schlaepfer et al., 2005; Rödl et al., 2007; Papacostas & Freestone, 2019), and often life-history traits, such as phenotypic plasticity (Prentis et al., 2008; Garnas et al., 2016) and *r*-selection (i.e. fast growth rates, high fecundity; Sakai et al., 2001; Jaspers et al., 2018) are also associated with invasive species.

Biological invasions have been responsible for major economic losses in industries such as agriculture, aquaculture, horticulture, and forestry (Pimentel et al., 2005; Bradshaw et al., 2016). The destructive effects of invasive species have also been major contributing factors to biodiversity loss and habitat destruction resulting in ecosystem change (Didham et al., 2005; Doherty et al., 2016; McClure et al., 2018). For instance, up to 40% of endangered species in the United States are threatened as a direct result of invasive species impacts (Pimentel et al., 2005), with climate change (Hellmann et al., 2008; Renault et al., 2018), and human-mediated land-use change exacerbating the issue (Vicente et al., 2011; Bullock et al., 2018).

The proliferation of invasive species is an ongoing issue accelerated by international trade and the movement of people (Hulme, 2009; Banks et al., 2015). This continues worldwide despite a more thorough understanding of the issue and increased implementation of stricter containment measures (Genovesi et al., 2015; Aizen et al., 2019). Over the last fifty years, a better understanding of the nature of invasive species spread has led to an increase in the use of biosecurity as a tool to prevent and/or contain invasive species (Hulme, 2010). Essentially,

biosecurity is a form of risk management that encompasses a range of disciplines, from ecology, biogeography and systematics to policy, social sciences and economics (Hulme, 2010; Richardson et al., 2010). Biosecurity is now an essential element of trade and international travel in most developed countries, with New Zealand being noted as a world leader in invasive species management and exclusion (Outhwaite, 2010; Molnar et al., 2008; Meyerson & Reaser, 2002).

1.1.2 Biological invasion definitions

The terminology used to classify and categorise biological invasions and invasive species is interchangeable and still subject to ongoing debate (Colautti & Richardson, 2009; Pereyra, 2016). The terms invasive/exotic/non-native/alien/unwanted and pest species, to name a few, are all used interchangeably in the published literature, often referring to the same types of species or similar scenarios (Falk-Petersen et al., 2006; Lockwood et al., 2013).

In this thesis I use the term 'invasive' species to refer to biological organisms that have already spread outside their native geographical or ecological range (*sensu* Valéry et al., 2008), and are known to, or are likely to, negatively affect the economy, the natural environment, or human wellbeing in the receiving environment (i.e. as per the International Union for the Conservation of Nature definition minus the term ‘alien’; International Union for the Conservation of Nature, 2017). One proviso I will add to this definition is that this can include native species. Native species have on occasion been characterised as invasive within their native environments (Valéry et al., 2009; Paine et al., 2011), usually as a result of some form of environmental change that has led to their proliferation and dominance in the ecosystem (Valéry et al., 2008). Dynamic population shifts are notable in bark beetles, and often impact how the species is described, e.g. as native, invasive or pest (Økland et al., 2009; Raffa et al., 2015; Seybold et al., 2016). Hence, in this thesis, the term invasive species can apply to native species if they exhibit invasive traits.

I use the term 'exotic' (in place of non-native or alien) to refer to an organism that is outside its normal geographic range, but where it is not known or assumed that it will cause detrimental impacts or not (as per Falk-Petersen et al., 2006). As this thesis is industry focused, I will also use the term ‘pest’ species where appropriate. This generally refers to a biological organism that is detrimental to an industry through economic losses (Falk-Petersen et al., 2006). In this instance, the origin of the species, i.e. whether it is native or exotic, is not defined (Falk-Petersen et al., 2006).

1.2 Forestry pests

1.2.1 Overview of invasive forest pests

The importation of wood and plant products is a major vector pathway for forest pests (Hulme, 2009; Roy et al., 2014; Hurley et al., 2016). Thousands of invasive species introductions have resulted from the trade in wood-based plant products such as raw logs, firewood and wood-packaging material (Meurisse et al., 2019). In Europe, 38% of introduced pests of plants have arrived through the horticultural trade, and this is predicted to increase as demand for products increases (Roques et al., 2009). Similarly, in Great Britain, 90% of introduced plant pests were likely a result of the plant trade, with woody exotic plants accounting for a significant proportion of introductions (Smith et al., 2007); and in the USA trade-introduced invasive wood boring insects have been intercepted in the thousands, with 25 different exotic Coleoptera becoming established during the period 1985-2005 (Haack, 2006).

Although this establishment number may not seem high, a single invasive forest species, once it is established, can cause extensive ecological and financial damage (Koch et al., 2011). Notable invasive forest pests such as the emerald ash borer (*Agrilus planipennis*) have accounted for widespread forest damage in North America (Herms & McCullough, 2014), killing an estimated 53 million ash trees (*Fraxinus* sp.) (Kovacs et al., 2010). It was estimated that damage and loss from this insect alone could cost a minimum of $10 billion over a 10 year period (Kovacs et al., 2010). Similarly, in China the red turpentine beetle (*Dendroctonus valens*) killed over 10 million *Pinus* sp., resulting in altered ecosystem function, loss of carbon sequestration, and a reduction in biodiversity (Lu & Sun, 2017). These are just two examples of an ongoing and increasing global problem of introduced invasive forest pests.

1.2.2 Bark beetles

Bark beetles (Coleoptera: Curculionidae) are considered one of the most successful invasive insect groups (Chase et al., 2017; Yu et al., 2019; Faccoli et al., 2020). This is partly due to their cryptic nature and their dispersal abilities, which result primarily from the widespread trade in host plant species (Kirkendall & Faccoli, 2010; Rassati et al., 2016; Chase et al., 2017). They are characterised as having an endophagic lifestyle whereby they live and feed within the cambium of trees (Price et al., 2011). In their native range bark beetles play a critical role in forest ecosystems where they predominantly facilitate in the decomposition process of the forest (Jenkins et al., 2008). Bark beetles can be classified as secondary or primary; primary

bark beetles attack healthy trees whereas secondary bark beetles colonise recently felled, and/or dead or dying trees (Pane et al., 1997). Primary species are usually only destructive when population numbers explode and aggregation pheromones attract large numbers of beetles (Raffa et al., 2015). Most species of bark beetles are secondary. These species are integral to the decomposition process, facilitating the break-down of wood via tunnel boring and thus allowing the nutrients to be cycled through the environment (Raffa et al., 2015). They also facilitate the entry of other invertebrates, fungi and micro-organisms into the wood through their tunnelling (Raffa et al., 2015). Because they are able to kill healthy live trees, primary bark beetles are considered more destructive than secondary in an invasive species context (i.e. some *Dendroctonus* sp. and *Ips* sp., Pane et al., 1997). However, secondary bark beetles can also become primary outside of their native range (Faccoli et al., 2020).

1.3 Commercial forestry

1.3.1 Global forestry

Global consumption and trade of forestry products have been steadily increasing (Food and Agriculture Organization, 2018). One unanticipated caveat to this trend has been the recent advent of the novel coronavirus disease (COVID-19, World Health Organisation, 2020), which has resulted in major declines in both imports and exports of forestry products worldwide (Food and Agriculture Organization, 2020). Forestry products cover a broad range of items from the least processed i.e. whole unprocessed logs and firewood, to more processed products, such as finished paper products, veneer/panels, and wood pellets (Allen et al., 2017; Food and Agriculture Organization, 2018). Sawn wood was the highest traded forestry product in 2018 (155 million m^3), followed by logs (138 million m^3), and then fibre furnish, which refers to the fibrous content of wood used to make paper products (117 million tonnes) (Food and Agriculture Organization, 2018). The trade of whole logs has been steadily increasing, with an increase of 7% between 2017-2018 (Food and Agriculture Organization, 2018); New Zealand (16%), Russia (14%) and the USA (9%) are the major exporters of whole unprocessed logs as of 2018 ((Food and Agriculture Organization, 2018).

1.3.2 New Zealand forestry industry

Pinus radiata (D.Don) or radiata pine is the primary forest species grown for timber production in New Zealand. Over 1.5 million hectares of radiata pine forests are planted throughout both the North and South Islands (New Zealand Forest Owners Association, 2019). Originally from

Monterey in California, hence the synonym Monterey pine, this species was brought to New Zealand circa 1859, originally for shelterbelts and firewood (Balchin, 2017), and then became used predominantly for forestry cultivation from the early 20th century onwards (Burdon et al., 2007; Burdon, 1992). The forestry sector in New Zealand currently employs over 38,000 people (Ministry for Primary Industries, 2020b), mostly from rural communities (Fairweather et al., 2000). As of June 2019 forestry contributed nearly $7 billion in revenue annually, providing 1.6% of the country's total gross domestic product (New Zealand Forest Owners Association, 2019). The majority of commercial forestry grown in New Zealand is exported to other countries, with exports to China accounting for over 70% of the export market; being shipped primarily as whole unprocessed radiata pine logs (New Zealand Forest Owners Association, 2019).

Although exotic pine forests in New Zealand are not environmentally neutral, they tend to have better environmental outcomes overall than other land uses such as dairy farming (Brockerhoff et al., 2008; Ausseil et al., 2013). As radiata pine is suited to more temperate climates it has flourished in New Zealand, and also in other Southern Hemisphere countries, where it is now recognised as being predominantly located despite its Northern Hemisphere origins (Burdon, 1992).

1.4 Market access

1.4.1 Overview

For a country to sell its goods internationally it must have entry to other countries' markets (Cook & Fraser, 2008). The term 'market access' defines this ability, with instruments such as trade agreements, tariffs, and policies regulating the entry of goods (Brenton-Rule et al., 2016). The accidental movement of exotic biological organisms is a likely outcome of trade (Hulme, 2009), with items such as plants, animals and their products considered particularly high risk (Hulme et al., 2008). As a result there are various international agreements and organisations responsible for regulating the movement of those trade products that have the potential to harbour exotic organisms (Brenton-Rule et al., 2016).

The World Trade Organisation (WTO) is the major governing body responsible for overseeing trade regulations between signatory countries (World Trade Organisation, 2014). Tariffs, trade agreements and non-tariff regulations are barriers for exporters to overcome, and the WTO's role is to ensure that these measures are not discriminatory or protectionist; they must be based

on current science and internationally agreed upon (World Trade Organisation, 2015). Heavily regulated non-tariff barriers (NTBs) such as those related to the Agreement on the Application of Sanitary and Phytosanitary Measures (the SPS Agreement) can be technically challenging to adhere to for some countries (Bourke, 1988; Henson & Loader, 1999). The SPS Agreement was implemented to protect countries against potential threats to human, animal, and plant health from the importation of diseases, pests or chemical residues associated with animals, plants, and their products (World Health Organisation, 1995). The export of plant products, such as radiata pine logs, is regulated under the International Plant Protection Convention (IPPC), which informs WTO member countries on best practice for managing phytosanitation through a set of standards - the International Standards for Phytosanitary Measures (ISPMs) (Food and Agriculture Organization, 2005). These standards are internationally agreed upon and comprehensive, covering everything from the implementation of a phytosanitary certificate system (ISPM 28; Food and Agriculture Organization, 2016) to the specific treatments and best practice for specific products e.g. ISPM 15 – treatment of wood packaging material.

1.4.2 Approved quarantine and pre-shipment (QPS) treatments for pine logs

The disinfestation of trade products is a relatively new phenomenon (Hulme, 2010). Disinfestation practices for logs prior to the implementation of the General Agreement on Trade and Tariffs (1947) policy were highly variable, with many countries requiring no treatments (Allen et al., 2017). The current standards to disinfest plant and forestry products are still variable depending on the exporting and importing country/region (Eschen et al., 2015). For instance, phytosanitary importing requirements (known as Importing Countries Phytosanitary Requirements - ICPR) for New Zealand export logs range in extreme from no requirements at all, i.e. no inspections, no pre-shipment treatment, no phytosanitary certification (IPCM Sri Lanka, Ministry of Primary Industries, 2014; IPCM Cook Islands, Ministry for Primary Industries, 2018a), to an extensive range of inspections, certification and pre-shipment treatments required (IPCM USA, Ministry for Primary Industries, 2017b; IPCM European Union, Ministry of Primary Industries, 2020a). The International Standards for Phytosanitary Measures (ISPM) 39 deals directly with the trade of wood products, with the most commonly adopted disinfestation strategies for logs being bark removal, fumigation, heat treatment/kiln drying and other chemical treatments (Food and Agriculture Organization, 2017). Irradiation and modified atmospheres are also listed as disinfestation methods. Nearly all disinfestation practices take place before transport i.e. quarantine and pre-shipment (QPS) (Food and Agriculture Organization, 2017), with chemical fumigation the most widely used and accepted

disinfestation method worldwide. Methyl bromide is considered the most important tool in this area (Pawson et al., 2019).

Fumigation has been the predominant QPS treatment for durable products such as logs and wood for several decades (Bond & Monro, 1984). Fumigation is a chemical treatment method, where a chemical is applied in gaseous form in order to deliver a lethal concentration of the chemical to the target organisms (Bond & Monro, 1984). Fumigation is different from other methods of chemical application, such as sprays or fogs, because fumigants are in gaseous form allowing the separated molecules to better penetrate durable materials (Banks, 1994). Fumigants also tend to diffuse relatively quickly after application, leaving minimal toxic residue, which makes fumigation a preferred method when controlling insects, fungi and microorganisms (Bond & Monro, 1984).

Fumigation with methyl bromide (MB) has been a widely accepted and internationally agreed-upon QPS treatment for export of radiata pine logs (United Nations Environment Programme, 2016b). However, as a move to reduce MB use, other QPS disinfestation strategies are now also being used and several more under are development (Pawson et al., 2014). Other treatments such as debarking (i.e. the removal of the bark), heat treatment (i.e. use of joule heating; Pawson et al., 2019), and phosphine (PH_3) are also utilised (Table 1; Ministry for Primary Industries, 2020b). Regardless of the treatment method, most countries require radiata pine logs to be pest-free, with several bark beetle species listed as unwanted by China, including *Hylurgus ligniperda* (F.) (Coleoptera: Curculionidae) (Ministry for Primary Industries, 2000).

Table 1. Outline of quarantine and pre-shipment (QPS) treatments required for radiata pine logs (*Pinus radiata* D.Don) prior to export to the top five export countries (by volume) from New Zealand.

Approved QPS method	Amount used (%)	Cost compared to MB	Treatment duration	Accepted import country
Methyl bromide (MB)	22[a]	-	~ 24 h	China India
Phosphine (PH_3)	77[b]	↓	~ 240 h	China
De-bark	8[b]	↑	-	China
*Heat treatment (≥ 56°C core temperature)	-	↑	30 min	India
†No specific treatment required	-	-	-	Japan Korea Singapore

[a]Ministry for Primary Industries, 2020c

[b]Yamoah & Glassey, 2020

*Not currently scaled-up for bulk treatments (Pawson et al., 2019)

†Product should still be pest free

Although there are a range of treatment options put forward by the IPPC, it is possible that the exporting country may not have the capability to carry out all QPS treatment options; or some may not be feasible for large volumes of logs (Methyl Bromide Technical Options Committee, 2018).

The fumigant PH_3 was approved as a QPS treatment for radiata pine logs exported to China from New Zealand in 2001 (Gear, 2004; Brash & Page, 2009). Log treatment with PH_3 is undertaken on board the ship whilst it is in transit. It is generated in-hold using aluminium phosphide, which reacts with moisture to create PH_3 (Hall et al., 2016). The application consists of 2 g/m^3 applied on day one, followed by a top-up of 1.5 g/m^3 at day five. The gas is then released after 10 days, prior to docking (Ministry for Primary Industries, 2018b). Subsequent modifications to the duration have recently been issued, with the duration extended for up to 15 days under cooler temperatures 10-15°C. Throughout the fumigation a minimum of 200 ppm of PH_3 must be maintained in the headspace atmosphere (Ministry for Primary Industries, 2018b). New Zealand's use of PH_3 for the disinfestation treatment of pine logs in-transit is a unique situation, with no other countries disinfesting whole logs in-transit in this manner for

export (United States Environmental Protection Agency, 2010; Armstrong, Brash, & Waddell, 2014).

1.5 Pine log disinfestation

1.5.1 Methyl bromide

Under the Montreal Protocol on Substances that Deplete the Ozone Layer (the Montreal Protocol) several ozone-depleting substances were identified, and a halt or phaseout of their use was agreed upon by the parties to the convention (United Nations Environment Programme, 2017). As a result, by 2016 over 98% of ozone-depleting substances have been phased out (United Nations Environment Programme, 2016a). Methyl bromide (MB) was and is still one of the substances targeted with annual reduction until it is completely phased out (United Nations Environment Programme, 2016a).

Prior to the Montreal Protocol, MB was predominantly used in agriculture to sterilise soil before planting (Ristaino & Thomas, 1996; Methyl Bromide Technical Options Committee, 2014). It was also heavily relied upon for post-harvest fumigation of crop plants (Carter et al., 2005), along with the sterilisation of a range of foodstuffs, commodities and structures (Noling & Becker, 1994; Taylor, 1994; Piccirillo & Piccirillo, 2010). MB is still in use under a critical use exemption (CUE), predominantly for quarantine and pre-shipment purposes (QPS) in situations where there are no other agreed upon viable alternatives (United Nations Environment Programme, 2018; Ministry for Primary Industries, 2019). Methyl bromide is a broad-spectrum fumigant that targets nematodes, insects, pathogens, fungi, and weeds (Piccirillo & Piccirillo, 2010). It dissipates quickly from the fumigated product, leaving less residue than other fumigants (Bond & Monro, 1984) and, as a result, it is less likely to seep into groundwater during soil fumigations (Noling & Becker, 1994). The qualities of MB have made it an efficacious and cost-effective fumigant, and it was the preferred method of sterilisation until the requirement arose for its subsequent phase-out (Ristaino & Thomas, 1997).

As a signatory to the Montreal Protocol, New Zealand has almost completely stopped using MB for any purpose except QPS (Ministry for Primary Industries, 2019a). Export logs and wood products now account for 94% of New Zealand's MB use (Ministry for Primary Industries, 2019a). Despite the gradual phaseout, the use of MB for QPS purposes has continued to increase sharply since the Montreal Protocol in New Zealand, due to an increase in the trade of goods requiring fumigation (Methyl Bromide Technical Options Committee, 2018). New

Zealand, in common with other countries, continues to research MB alternatives, although, finding a comparable alternative has proved challenging (Porter et al., 2009). By February 2022, New Zealand aims to achieve recapture of MB; containing/recovering any MB released so that it will not enter the atmosphere (Methyl Bromide Technical Options Committee, 2018; Environmental Protection Agency, 2021). In the meantime, other fumigants such as phosphine and ethanedinitrile are either in use in place of MB or under investigation to replace it (Fields & White, 2002; Ducom, 2012).

1.5.2 Phosphine - an alternative fumigant

Phosphine (PH_3) is a common commercial fumigant that has been in use for over 80 years, mainly for crop protection in the agricultural industry (Ryan & Lima, 2014). It is cost-effective, leaves minimal residue, is easily applied to a range of commodities, and is a globally accepted phytosanitary method (Nayak & Collins, 2008). With regulation leading towards a complete phaseout of MB, PH_3 is considered to be one of only a few viable alternatives currently available (Ducom, 2012; Ryan & Lima, 2014). PH_3 is not historically associated with disinfesting logs or wood, but it is successfully used for the treatment of insect pests in stored grain and other durable stored foodstuffs (Brash & Page, 2009; Glassey, 2009). Primarily based on its application for stored food, PH_3 has been developed as an alternative to MB as a phytosanitary treatment of New Zealand export pine logs against insect pests (Zhang et al., 2004; Armstrong et al., 2014).

Prior to 2001, when New Zealand starting using PH_3 for in-transit log fumigations, few peer-reviewed studies had been carried out on either the efficacy of PH_3 against pests of wood products or on its efficacy for use in wood disinfestation in general (Brash & Page, 2009). One study, addressing a similar export scenario to that of New Zealand, treated woodchips on-board with PH_3 en route from the USA to Sweden (Leesch et al., 1989). The target pest was the pinewood nematode (*Bursaphelenchus xylophilus*). It was found that PH_3 was 93% effective in controlling the nematode, with the remaining survivors noted as being located in cooler areas of the holds (~ 15-20°C) (Leesch et al., 1989). Later published studies have shown that PH_3 can be effective against pests of wood, and for wood disinfestation in general (e.g. termite control, Choi et al., 2014; powder post beetles: Bostrichidae, Pant & Tripathi, 2012; Remadevi & Deepthi, 2018). For example, testing of the New Zealand to China QPS treatment schedule under laboratory settings showed that the schedule was effective against the forestry pests *Hylastes ater* and *Arophalus ferus* (Zhang et al., 2004). Further, PH_3 penetrates radiata pine

well, particularly at lower temperatures (Hall et al., 2018), and quickly reaches equilibrium between wood and the headspace atmosphere compared to other fumigants (Ren et al., 2011).

Phosphine has a significantly slower action on insect pests compared to other fumigants, such as MB. Hence, PH_3 must be applied for days as opposed to hours (Fields & White, 2002), with application times needing to be extended under cooler conditions (Chaudhry et al., 2004). Low temperatures, even those considered temperate (~ 15°C) can markedly reduce PH_3's effectiveness due to a decrease in insect respiration (Chaudhry et al., 2004). One recommendation is to avoid using PH_3 altogether if the grain temperature is below 15°C (Warrick, 2011; Burrill et al., 2016). Several shortcomings have been noted with respect to PH_3 efficacy in the grain industry, such as ineffectiveness at low temperatures (Nayak & Collins, 2008), and lowered efficacy in low oxygen environments (Kashi, 1981). However, the principal issue in this industry is the development of resistance in some insect populations (Nayak et al., 2017). This has been linked to earlier misuse of PH_3, through poor/inconsistent application, leaky grain silos (Chadda, 2016), and/or sub-lethal concentrations (Shi et al., 2013) enabling resistance. For instance, it has been noted that resistant species need $\geq$ 28 days exposure, concentration dependent, to PH_3 to achieve efficacy (Collins et al., 2001; Nayak et al., 2020). In contrast, a duration of 7-10 days is required for less resistant species, varying by the target pest species and temperature (Warrick, 2011).

With regard to treating pine logs in transit with PH_3, resistance was thought to be able to be overcome due to the on-board in-transit nature of the fumigation (Glassey, 2009; Brash & Page, 2009). Insect escape is not possible at sea, nor is there a need for repeated applications over a long period of time (Glassey, 2009). Further, this unique situation of in-transit fumigation allows for comparable fumigation times and doses to that used in the grain industry (Glassey, 2009); hence the issue of insect resistance is mitigated by off-shore, in-transit fumigation.

1.5.3 Phosphine mode of action upon insects

Phosphine (hydrogen phosphide, PH_3) is a colourless, odourless gas in its pure form, and has a similar density to air, thus it is easy dispersed (Bond & Monro, 1984; Chaudhry, 1997). As it is a strong reducing agent (i.e. electron donating), PH_3 can oxidise to produce oxy-acid products, which are reactive to copper and other metals (Chaudhry, 1997). PH_3 is highly flammable and can auto-ignite when it is concentrated (> 1.7%) (Nath et al., 2011; Food and Agriculture Organization, 2019). PH_3 poisoning results from inhalation or ingestion; it does not penetrate the skin or integument (Bond & Monro, 1984). PH_3 is considered one of the most

potent invertebrate poisons, however at concentrations used for non-perishable commodities, it is slow acting (Bond & Monro, 1984; Schlipalius et al., 2018). Due to the slow mode of action by PH_3 there is not usually a simplified linear relationship between the parameters of PH_3 dose (*C*) and time (*t*) to create a single constant (*k*) (Winks, 1986; Annis, 1999). In a linear relationship, the concentration of a toxin and the time exposed to that toxin are thought to be equally effective in killing the target species (see Haber's Rule, Witschi, 1999), hence a half concentration for double the time will be as effective as a full concentration for half the time (Winks, 1986). However, this is not the case for PH_3 use in non-perishable products; instead, time is the pertinent variable, particularly against insect pests and concentration plays a lesser role (Hole et al., 1976).

Phosphine's mode of action on insects begins when it enters the spiracles through active respiratory uptake (Chaudhry, 1997). Therefore PH_3 uptake and efficacy are dependent on aerobic respiration; PH_3 has minimal effect on anaerobic respiring organisms (Nath et al., 2011). Once PH_3 enters an insect's body its precise mode of action is unclear (Chaudhry, 1997; Nath et al., 2011). One proposed mechanism is that PH_3 causes oxidative stress to biological macromolecules through the creation of reactive oxygen species (ROS) (Nath et al., 2011; Ma et al., 2017). This leads to cell death through oxidative stress (Leelaja & Rajini, 2012), by way of lipid peroxidation within the cell membrane (Schlipalius et al., 2018). This 'oxidative stress' pathway has been illustrated in resistant species (Schlipalius et al., 2018). For instance, some resistant grain pest species have genetic variants *rph1* and *rph2*, which have been identified as PH_3 resistant phenotypes (Schlipalius et al., 2002; Schlipalius et al., 2012). These variants are thought to reduce the impacts of ROS from PH_3 treatment, as the desaturation of fatty acids is likely decreased in these phenotypes, thus reducing lipid peroxidation (Schlipalius et al., 2018).

Another potential PH_3-mediated stress is metabolic shutdown through the disruption of oxidative phosphorylation, leading to an inhibition of oxidative respiration and ATP production (Corrêa et al., 2014). PH_3 strongly inhibits some biological redox systems such as mitochondrial complex IV (cytochrome *c*) (Corrêa et al., 2014). The resulting disruption to cytochrome *c* causes an insufficiency of energy, leading to aerobic metabolic shut down and, eventually, to death (Corrêa et al., 2014). Resistance to PH_3 toxicity via hypometabolism (low metabolic rate) is congruent with this pathway and has been demonstrated using a PH_3 resistant *Caenorhabditis elegans* strain as a model organism (Ma et al., 2017). Resistant *C. elegans* showed a 25% reduction in metabolic rate compared to non-resistant conspecifics (Ma et al., 2017). This is also consistent with the theory that PH_3 efficacy is closely related to insect

respiration and uptake, with insects that display comparatively lower aerobic respiration also showing resistance (Nath et al., 2011).

1.6 Insect gas exchange

1.6.1 Overview

Insect gas exchange requires the uptake of oxygen (O_2) and the expulsion of carbon dioxide (CO_2), like most animals (Chown & Nicolson, 2004; Matthews & Terblanche, 2015). However, the insect respiratory system is markedly different when compared to more familiar vertebrate systems (Matthews & Terblanche, 2015). O_2 enters the insect's body through the spiracles, which are lateral circular openings along the body. From the spiracles, O_2 is diffused throughout the body via a dense branching tracheal system. O_2 is supplied directly to the cells via fluid-filled tracheoles that are smaller (< 1 µm in diameter) trachea branching off from the larger tracheal system. CO_2 is able to exit via the same pathway in reverse (Matthews & Terblanche, 2015) (Fig. 1). This type of respiratory system is characterised as 'open' and 'direct'. There is no intermediary circulatory system responsible for the movement of O_2 and CO_2, and O_2 is received directly by the mitochondria (Matthews & Terblanche, 2015). Smaller insects, and insect eggs, likely rely solely on the passive diffusion of O_2 from the spiracles to tracheole (Lighton, 1988; Terblanche & Woods, 2018; Woods & Hill, 2004). But insects can and do employ a range of mechanisms to modulate their O_2 consumption (Lighton, 1996).

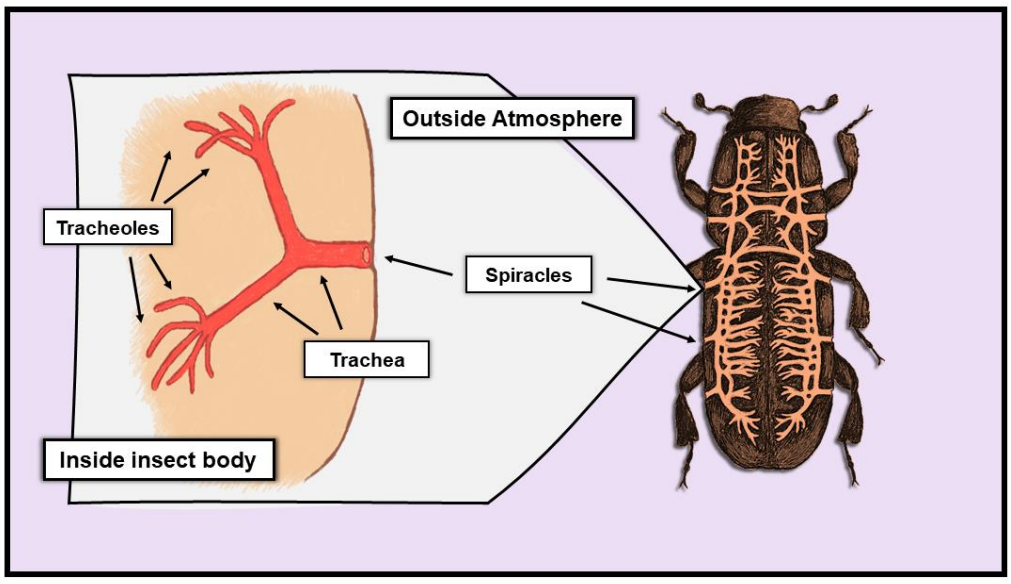

Fig. 1 Simplified diagram of the insect respiratory system. Illustration, Devitt, 2020.

Insect respiratory patterns are highly diverse, with three broad categories: cyclic, where there is a regular release of CO_2 and no prolonged spiracle closure; continuous, where CO_2 is released continuously and there is no observable cycle or pattern; and discontinuous gas exchange (DGC), where there is a specific gas exchange pattern exhibited in three phases (Fig. 2) (Gibbs & Johnson, 2004; Terblanche & Woods, 2018).

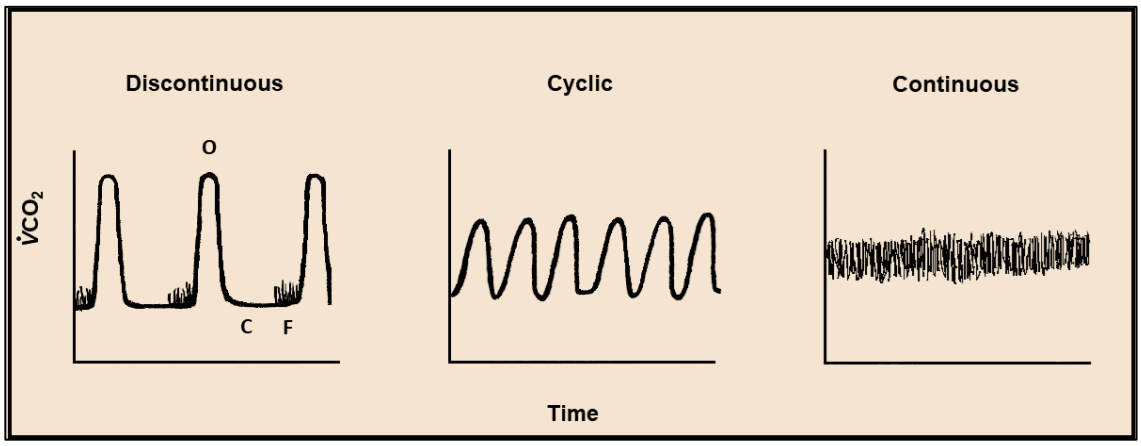

Fig. 2 Insect gas exchange patterns from **left to right**: discontinuous gas exchange (DGC) showing the 'closed' phase (C), 'open' phase (O), and 'flutter' phase (F). Cyclic gas exchange, and continuous gas exchange. Illustration, Devitt, 2020.

In DGC, the first phase is 'closed', where the spiracles are closed and no gas exchange is taking place; the second phase is the 'flutter' phase, where the spiracles quickly open and close; the last phase is the 'open' phase, where the spiracles open and a burst of CO_2 is released (Lighten, 1996; Matthews, 2018). Put simply, this pattern is alternating between periods of restricting O_2 uptake (i.e. breath-holding) followed by a sudden discharge of CO_2 (Matthews, 2018). There are several competing hypotheses around why DGC is observed in some insect species: the hygric hypothesis suggests it is a mechanism to restrict water loss (Kestler, 1984; Schimpf et al., 2009); the chthonic hypothesis posits it is an adaptive strategy in response to low O_2 and high CO_2 environments (Lighton, 1998; Lighton & Joos, 2002); and the oxidative damage hypothesis argues it is an adaptation to minimise oxidative damage (Hetz & Bradley, 2005). These hypotheses and others not mentioned (for overview see Chown et al., 2006; Terblanche & Woods, 2018), have all been hotly contested; the adaptive need for DGC remains largely unresolved.

An additional complexity in insect gas exchange is that some insects display all three different gas exchange patterns (e.g. cyclic, continuous, and DGC) under different physiological states,

and life stages (Terblanche & Woods, 2018). DGC appears to have evolved independently in five different insect orders, which suggests that it is an adaptive strategy (Marais et al., 2005). There is generally a lack of study in the area of insect gas exchange patterns, with an apparent focus on DGC rather than other respiratory patterns (Quinlan & Gibbs, 2006). This may be because of the 'file-drawer problem' where only significant studies are published (Csada et al., 1996). For instance, studies which demonstrate DGC are more likely to be published whereas studies that do not identify DGC are considered non-significant and are never published, hence left in a 'file-drawer' (Marais et al., 2005; Quinlan & Gibbs, 2006). Likely there are numerous insect gas exchange patterns, which are employed for a range of reasons. The more insect species gas exchange patterns that are analysed and reported, the more we can build knowledge in this depauperate area (Terblanche & Woods, 2018).

1.6.2 Adaptation to hypoxia

Insects display a wide range of tolerance to low O_2 (hypoxic) and high CO_2 (hypercapnic) atmospheres (Chown & Nicolson, 2004; Levy-De la Torre et al., 2019). On the extreme end, some insect species can last weeks without any O_2 at all (anoxia) (Hoback, 2012). For instance, tiger beetle larvae, *Cicindela togata* (Cicindelidae, Coleoptera), can survive over 100 hours in anoxic (0% O_2) conditions (Hoback et al., 1998). Some insect species are adapted to live in hypoxic environments (Hoback & Stanley, 2001; Zhang et al., 2017). Endophagic insects that reside in enclosed spaces, such as insects that tunnel, bore, mine and form galls have evolved in a naturally hypoxic/hypercapnic environment compared to the outside ambient atmosphere (Woodman et al., 2007; Hoback, 2012). There are several factors associated with these types of environments that make them hypoxic/hypercapnic. There is likely a decrease in O_2 diffusion within an often tight, small space (Pincebourde & Casas, 2016). Further, the substrate that the insect is in will likely alter the atmospheric conditions by harbouring respiring bacteria or presence of other organisms, as well as its own physiological processes, which reduce available O_2 and add CO_2 (Lighton, 1998; Pincebourde et al., 2006). An insect's capacity to withstand hypoxia is highly variable and changes throughout its lifecycle (Harrison et al., 2018).

Tolerance to hypoxia is also associated with tolerance to low temperatures (Boardman et al., 2015). An extreme example is the alpine beetle *Melasoma collaris* which can survive anoxic atmospheric conditions for 120 days at 0°C (Meidell, 1983). *M. collaris's* extremely long survival time under anoxia has potentially evolved because of exposure to anoxic conditions,

while encased within ice during the winter months, in conjunction with a reduction in metabolism from the cold temperatures (Meidell, 1983).

Cross-tolerance from hypoxia has influenced outcomes for pest control techniques. Cross-tolerance is a phenomenon where tolerance or resistance to a stressor is induced as a consequence of being exposed to another stressor (Boardman et a., 2015). For example, irradiation (Hallman, 2004; Wang et al., 2019), PH_3 (Nath et al., 2011), and cold disinfestation treatments (Boardman et al., 2015) are less effective if the insect is preconditioned under hypoxia prior to or during treatment. Conversely, heat treatments applied under hypoxic atmospheres can be more effective than heat treatment alone (Boardman et al., 2018). It is thought that the stress mechanisms that are elicited in response to a hypoxic atmosphere (e.g. hypoxia-inducible factor 1; Wang et al., 1995; Zhao et al., 2012) infer oxidative protection in some species (Boardman et al., 2018; Cao et al., 2019). Hence control methods that directly impact the insect via the oxidative stress pathway have been shown to be less effective in hypoxia adapted or pre-conditioned insects (Cao et al., 2019). For instance, irradiated Caribbean fruit fly (*Anastrepha suspensa*) showed significantly higher survival rates, and increased fitness, when exposed to anoxic conditions before radiation treatment (López-Martínez & Hahn, 2012). *A. suspensa* was shown to have an increased antioxidant capacity as a result of the anoxia pre-conditioning. Similarly, PH_3 is thought to be less effective on insect pests that have a stronger capacity to detoxify or protect against ROS (Nayak et al., 2020). There is little literature specifically on the hypoxia/hypercapnia tolerance of endophagic insects that reside within plant tissues, such as bark beetles (Pincebourde & Casas, 2016). Further, there appears to be no literature currently on potential atmospheric conditions within the tunnels made by phloeophagous (feeding on phloem) and xylophagous (feeding on wood) beetles (Pincebourde & Casas, 2016). It is unknown how these potential adaptations influence pest control.

1.7 Study organism: *Hylurgus ligniperda*

Hylurgus ligniperda (F.) are phloeophagous bark beetles that consume the cambium or inner phloem of *Pinus* sp. (Raffa et al., 2015). They are native to Europe, parts of Asia and the Middle East (Wood & Bright, 1992). However, *H. ligniperda* has extended its range to North and South America, Oceania, Spain, Japan, Sri Lanka, and South Africa (Faccoli et al., 2020); most likely through hitchhiking on wood products (Haack, 2006). *H. ligniperda* is a secondary bark beetle species; it does not generally attack live trees but instead will colonise cut fresh logs, stumps

and slash (Haack, 2006; Meurisse & Pawson, 2017). *H. ligniperda* is considered a forestry pest and wood exports must be free of *H. ligniperda* for most countries (Pawson et al., 2014; Faccoli et al., 2020). Although *H. ligniperda* does not generally attack healthy living trees, it is a known vector of several pathogenic fungi (i.e. Ophiostomatoid spp.) (Davydenko et al., 2014), which can stain wood, reducing its sale price (Thwaites et al., 2005). Other fungal species, (*Leptographium* spp.) linked to root disease in several *Pinus* species have also been found on *H. ligniperda* (Kim et al., 2011; Davydenko et al., 2014).

Adult *H. ligniperda* are 4-6 mm in length and 2 mm in width, with the fully developed adult dark brownish-black in colour; teneral adults are initially a light tan colour, and subsequently darken with age (Kimoto & Duthie-holt, 2006). A distinguishing feature of *H. ligniperda* is the reddish-brown hairs covering the elytra and the posterior slope, where they are more noticeable (United States Department of Agriculture, 2002). Sexual dimorphism is evident in many bark beetle species, however in *H. ligniperda* morphological sexual dimorphism is not readily apparent (Liu et al., 2008). The males of this species stridulate as a form of courtship, as do other species in this family (Kirkendall et al., 2015). No sex aggregation pheromones have been noted in *H. ligniperda*, thus mating on new hosts is thought to occur by chance (Chase et al., 2017). Gravid females create branching arms off from the main gallery where they lay each egg in a single niche. Hatched larvae feed on the available phloem for several weeks before pupating (Raffa et al., 2015). Newly emerged adults leave the host to search out new hosts and mate in spring/summer (Kerr, 2010; Mausel et al., 2007) (Fig. 3).

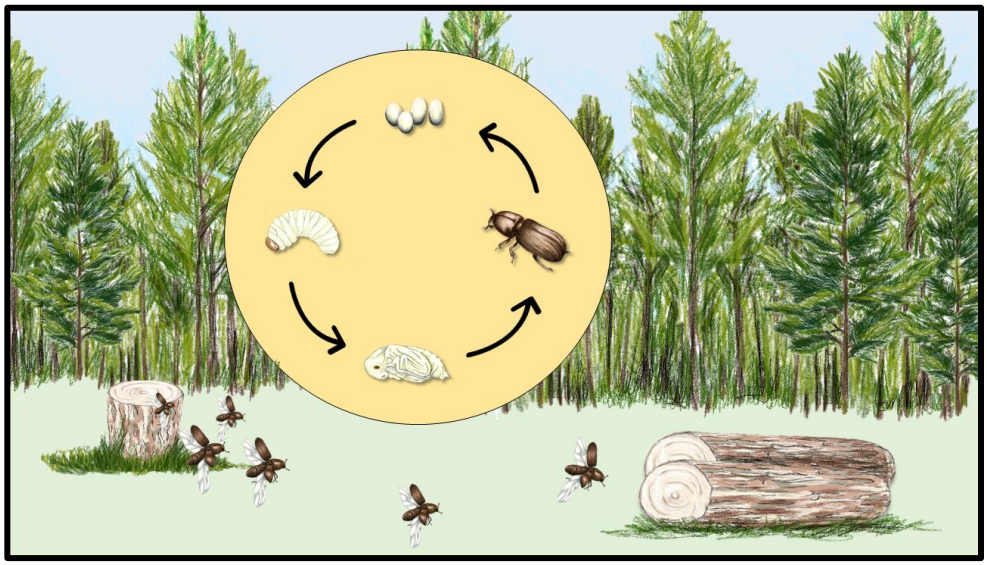

Fig. 3 Diagram of *Hylurgus ligniperda* lifecycle. Adult beetles infest stumps and felled logs, where they mate and complete their life cycle (yellow circle). Illustration, Devitt, 2020.

Hylurgus ligniperda is widespread throughout New Zealand and is a pest of significance in the export of New Zealand pine logs (Brockerhoff et al., 2006). It is noted as an unwanted pest particularly by New Zealand's largest trade partner, China, where it has a high chance of establishing (Ministry for Primary Industries, 2000; Yu et al., 2019). Further, *H. ligniperda* has been intercepted at the Chinese border on New Zealand pine exports (Zhang et al., 2004; Ministry for Primary Industries, 2015).

1.8 Research aims

The overarching aim of this PhD thesis was to establish the efficacy of phosphine (PH_3) against bark beetles, using *Hylurgus ligniperda* as the study species. I sought to untangle some of the different and complex elements that may be present during the in-transit PH_3 fumigation of radiata pine logs, which could impact its efficacy. Using a range of experiments I sought to answer the following questions: 1) How does the respiration rate of contained radiata pine logs modify the surrounding atmosphere during transit, 2), What are the respiratory patterns of *H. ligniperda* under different atmospheric conditions, and 3), What is the effective dose range of PH_3 to achieve 100% mortality for *H. ligniperda* over all four of its life stages?

1.9 Thesis layout

This thesis consists of three data chapters (Chapters 2-4) prepared as stand-alone research papers. Chapter 2 is a collaborative joint-author paper, so is written using the first-person plural. Chapter 3 is also written using the first-person plural as this was also a collaborative project, intended for publication. Chapter 4 is written in the first-person singular as it was primarily my own work that contributes to a larger programme of work so is not intended for publication as it stands. The final chapter (Chapter 5) synthesises the results of this study and recommends future research.

Chapter 2 The aim of this study was to determine how pine log respiration altered atmospheric conditions in a ship's hold. We quantified the amount and rate of carbon dioxide (CO_2) produced and oxygen (O_2) depleted by radiata pine (*Pinus radiata* D.Don) logs in airtight containers under controlled laboratory conditions at different temperatures (10, 15 and 20°C) and in different seasons i.e. spring, summer, autumn and winter. We hypothesised that CO_2 production by radiata pine logs would increase as temperature increases, and concurrently that O_2 would decrease. We also hypothesised that season would influence the amount and rate of CO_2 production. If true, we predicted that radiata pine logs harvested during the peak growth seasons (spring and summer) would produce the most CO_2 because their growth rate is faster in warmer months than during slow growth, cooler months (autumn and winter). This information will enable us to quantify the changing levels of CO_2 and O_2 that insects may be exposed to at different temperatures during commercial in-transit insect control.

This paper is published:

Devitt, J. C., Hall, M. K., Najar-Rodriguez, A. J., & Beggs, J. R. (2020). Respiration by unprocessed pine logs creates a severe hypoxic and hypercapnic atmosphere in enclosed spaces. *Journal of Stored Products Research*, *88*, 101670.

Chapter 3 Our aim in this study was to assess the respiration physiology of bark beetles using *Hylurgus ligniperda* (F.) as our model. We measured the respiration rates and the critical oxygen threshold limit (P_{crit}) of three life stages of this beetle. We used stop-flow respirometry to ascertain the respiration of *H. ligniperda* larvae, pupae, and adults under three O_2 treatments: hypoxia (2 kPa), normoxia (20.9 kPa), and hyperoxia (40 kPa). We included two temperatures 10°C and 20°C, and two humidity variables – dry (0% RH) and wet (95% RH). We then used flow-through respirometry to assess how tolerant the three *H. ligniperda* life stages were to low O_2 environments, using a stepwise reduction in O_2 (20.9-0.5 kPa) in order to establish the lowest level of O_2 this species could tolerate before metabolic shut-down occurred.

We predicted that all life stages of *H. ligniperda* would be tolerant to severe hypoxia (≥ 2% atmospheric O_2) and that respiration would vary with life stage. We predicted that pupae would be the most tolerant to hypoxia because this life stage likely had a dormancy response to low atmospheric O_2. We hypothesised that metabolic rates of all life stages would decrease as temperature, humidity and atmospheric O_2 decreased.

Chapter 4 In this study I sought to evaluate the efficacy of PH_3 treatment of pine logs against bark beetles. I aimed to establish a clear dose-response relationship between the toxicity of PH_3 to the response of *H. ligniperda* at both 10°C and 20°C. Firstly, a series of 72-hour rangefinder fumigations were undertaken to establish which PH_3 concentrations to test against exposed (removed from cambium) life stages of *H. ligniperda*. I then undertook longer duration experiments (120-240 hours) as initial 72-hour experiments failed to achieve a clear dose-response relationship. Additional rangefinder experiments were undertaken, which fumigated insects that were still within the cambium (in-log) to assess PH_3 efficacy in a more realistic scenario, however again a clear dose-response relationship was not established. Finally, I tested the already established commercial fumigation schedule for PH_3 treatment of pine logs under what were deemed favourable conditions (hereafter the commercial scenario) based on my earlier experiments.

Chapter 2 - Respiration by unprocessed pine logs creates a severe hypoxic and hypercapnic atmosphere in enclosed spaces

2.1 Introduction

Effective phytosanitation of wood products, particularly raw unprocessed wood, is paramount in controlling the spread of unwanted biological organisms, as well as minimising trade barriers (Liebhold et al., 2012; Piel et al., 2008). To mitigate the spread of unwanted insects, quarantine and pre-shipment (QPS) phytosanitation treatment is a requirement for most countries before wood can be exported (Schrader & Unger, 2003). Despite this, worldwide transportation of wood products has spread insect pests, such as the Asian long-horned beetle *Anoplophora glabripennis*, and the woodwasp *Sirex noctilio*, that cause extensive economic and ecological damage (Corley et al., 2019; Lantschner et al., 2017; Meurisse et al., 2019). New Zealand has a large export industry for radiata pine (*Pinus radiata* D.Don), particularly for unprocessed logs, which account for 55% of total wood exports (Ministry for Primary Industries, 2019b), most of which are exported to China (New Zealand Forest Owners Association, 2017). Methyl bromide (MB), phosphine (PH_3) (applied in-transit as aluminium phosphide) or debarking are applied as approved QPS treatments in New Zealand against forestry pests infesting these logs (Table 1).

Table 1. Approved quarantine and pre-Shipment (QPS) treatments for *Pinus radiata* (D.Don) exported from New Zealand to China (Ministry for Primary Industries, 2017a). Methyl bromide is applied dockside before transport. Phosphine is applied in-hold, during transit.

Approved quarantine and pre-shipment treatments: China		
Debarking	**Methyl bromide**	**Phosphine**
< 5% bark cover per single log < 2% bark cover per log stack	80 g/m^3 for 16 hours ≥ 15°C 120 g/m^3 for 16 hours for 5-15°C	2 g/m^3 initial dose 1.5 g/m^3 at day 5 Fumigant released after 10 days

Methyl bromide is a widely accepted QPS treatment for export wood products. It provides effective control under a wide range of conditions against a range of pests (Noling & Becker,

1994; Ristaino & Thomas, 1997). However, the release of MB into the atmosphere is being phased-out as it depletes the ozone layer (United Nations Environment Programme, 2018). Several alternative fumigants, such as ethanedinitrile (Pranamornkith et al., 2014), sulfuryl fluoride (Barak et al., 2010) and PH_3 (Zhang et al., 2004) have shown promise for replacing MB in the disinfestation of wood products. Fumigant efficacy is closely related to insect respiration and metabolism, hence efficacy can be variable depending on abiotic conditions as well as the respiration biology of the insect species (Liu, 2011; Nayak & Collins, 2008).

High CO_2 (hypercapnic) and low O_2 (hypoxic) atmospheres have been recorded in ship cargo holds during the mass transport of logs and wood products, predominantly concerning workplace health and safety (Svedberg et al., 2008). Cellular respiration continues in moist cut wood (Zabel & Morrell., 1992), which can modify the surrounding atmosphere. Microbial organisms associated with wood also respire, adding to a reduction in O_2 and an increase in CO_2 (Svedberg et al., 2008). Atmospheric levels of CO_2 can reach levels fatal to humans (> 10%) within 48 hours of wood storage, and O_2 levels can also be severely reduced within this timeframe (< 6%) (Svedberg et al., 2009). Further, the more 'raw' or unprocessed a wood product is, the faster the change in the atmosphere potentially due to high levels of microbial respiration (Svedberg et al., 2009).

Generally, hypercapnic and hypoxic atmospheric levels are deleterious for most animals, including insects (Harrison et al., 2018). However, for some insects, exposure to low O_2 levels has been linked to cross-resistance to some control methods, such as radiation (Hallman, 2000), fumigants (Bond et al., 1967), and low temperature sterilisation (Boardman et al., 2015). A reduction in insect respiration has been linked to a reduction in the efficacy of several fumigants (Bond, 1963). For instance, the efficacy of PH_3 against the grain pests grain weevil *Sitophilus granarius* and wheat beetle *Tenebroides mauritanicus* greatly decreased depending on the O_2 level in the fumigated atmosphere. The survivorship of the pests increased when they were exposed to a low O_2 environment before fumigation, compared to a decrease in tolerance when exposed to a high O_2 environment before fumigation (Bond, 1963). Similarly, creating a hyperoxic environment (> 40% O_2) during fumigation can increase PH_3 toxicity significantly, as compared to ambient O_2 levels (20.9%) (Liu, 2011).

Environmental conditions within a relatively airtight ship hold could affect log respiration and therefore influence the efficacy of some insect control treatments applied during transit. The respiration of logs in this environment is complex and might be influenced by a range of

potentially interacting factors such as fluctuating diurnal and seasonal temperature, and variable moisture levels depending on bark cover, tree age, time from harvest and season. Thus, quantifying how atmospheric conditions change because of log respiration is therefore critical for evaluating and improving forest biosecurity.

In this study we quantified the amount and rate of CO_2 produced and O_2 depleted by radiata pine logs in airtight containers under controlled laboratory conditions at different temperatures (10, 15 and 20℃) and in different seasons i.e. spring, summer, autumn and winter. We hypothesise that CO_2 production by radiata pine logs will increase as temperature increases, and concurrently that O_2 will decrease. We also hypothesise that season will influence the amount and rate of CO_2 production. If true, we predict that radiata pine logs harvested during the peak growth seasons (spring and summer) will produce most CO_2 because their growth rate is faster in warmer months than during slow growth, cooler months (autumn and winter). This information will enable us to quantify the changing levels of CO_2 and O_2 that insects may be exposed to at different temperatures during commercial in-transit insect control. This will also provide valuable detail on the magnitude and timing of these atmospheric changes to assess the potential impact on insecticide efficacy and to help identify ways that insect control could be improved.

2.2 Methods

2.2.1 Trees

We sourced radiata pine trees from a commercial farm in Bulls, Manawatu, New Zealand (40°11’21”S 175°20’31”E) between 2017 and 2018, in austral spring, summer, autumn and winter. The farm/forest is a 260-ha block with 110 ha of production forestry, predominantly radiata pine. High pruning was undertaken when the trees were aged between 7-8 years, and then a year later thinned from 1000 stems per ha to 400 stems per ha, and finally a production thin to 200 stems per ha. This is a lower stocking rate than other typical foresters (D. Hocking personal communication, May 2019). Radiata pine trees used for the experiments were planted approximately 10 years ago, and at time of harvest had a mean diameter of 237.1 ± 1.7 mm at chest height.

We cut the logs into 175-280 mm sections based on their respective diameters. This was to achieve a container loading factor (volume/volume % - the proportion of the container occupied by the volume of the commodity) of between 40-50%, to simulate the commercial in-hold

loading factor of ~ 50% (Stakeholders in Methyl Bromide Reduction Incorporated, 2018). However, in practice, the average loading factor achieved in the experiment was 41.2 ± 0.7% due to the size constraints of the containers (Fig. 1).

Trees were felled by chainsaw (not commercially harvested) on the same day in the middle of each of the four seasons: austral spring (October), summer, (January), autumn (April), and winter (July). Ten individual trees within the diameter range were selected from the same 15 ha plot for each season, and three log sections were cut from each tree, giving a total of thirty log sections per season. Log sections maintained full bark coverage after cutting, reflecting the situation of commercial harvesting scenarios.

We randomly assigned log sections to one of the three temperature groups 10, 15 or 20°C. Each temperature group had 10 logs, representing biological replicates. The logs were acclimatised at their respective temperature for 24 hours before the experiment commenced, then weighed and measured before being enclosed within the airtight containers (345 mm x 300 mm x 270 mm; 20-L container polypropylene with UV additive, Mitre 10™).

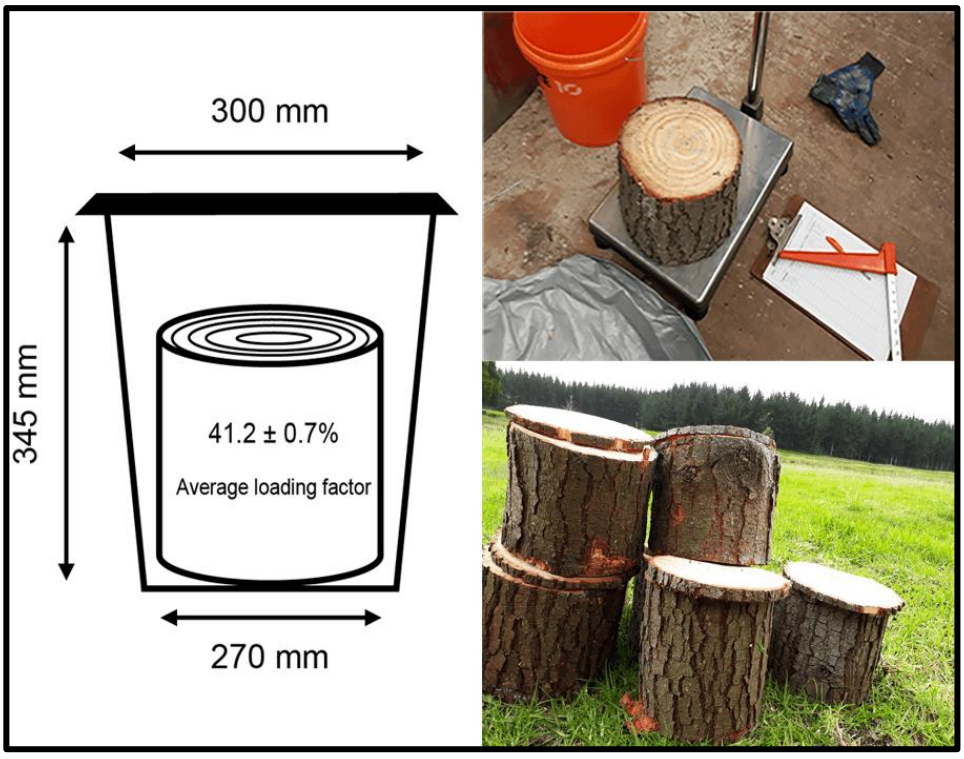

Fig. 1 Left: diagram showing log placement within container, **top right:** weighing and measuring logs before placement in container, **bottom right:** example log sections with their respective slices for moisture content analysis. Loading factor is the proportion of the container occupied by the volume of log.

Fifty mm slices from the log sections (Fig. 1) were weighed (Mettler PC 8000) before going into a temperature-controlled cabinet (Marford, GEO Wilton & Co. Ltd, Wellington, New Zealand) set at 103 ± 2°C for 96 hours. Logs were then weighed again to ascertain the dry weight (Fig. 1). Percentage moisture was calculated using the following equation:

$$\textbf{\textit{Moisture content \%}} = \frac{A-B}{B} \times 100$$

Where *A* is the wet wood slice and *B* is the dry wood slice (ASTM International, 2016). The average percentage moisture content for the logs harvested was; 135.6 ± 2.8% for spring, 143.1 ± 3.5% for summer, 129.3 ± 3.0% for autumn, and 108.4 ± 3.9% for winter.

2.2.2 Gas Chromatography

We sealed logs inside 20-L airtight containers and took a headspace sample from each container within the first 2 hours to assess initial CO_2 and O_2 levels using gas chromatography. The containers were housed in temperature-controlled shipping containers (Transicold Thinline) at either 10 ± 1°C, 15 ± 1°C or 20 ± 1°C for 14 days.

We measured CO_2 and O_2 concentrations using an SRI 8610C (Mandal, USA) gas chromatograph (GC) fitted with a thermal conductivity detector (TCD) with a CTR-1 packed column (1,828.8 mm x 6.35 mm, 9' MS5A and 6' Hayesep D). The GC oven temperature was isothermal set to 50°C, and the TCD was set to 175°C. The carrier gas was helium (BOC, New Zealand, instrument grade > 99.99%) set to 60 psi, hydrogen (BOC, New Zealand, instrument grade > 99.98%) 25 psi, air (BOC, New Zealand, dry, O_2 21.0 ± 1.0% in nitrogen balance) 2 psi. Total runtime per sample was 3 minutes, with an approximate retention time of 50 and 70 seconds for CO_2 and O_2, respectively. A weekly three-point CO_2 and four-point O_2 calibration was done using CO_2 and O_2 gas standards; 0%, 0.5% (balance in nitrogen) and 20% (balance in air) CO_2, and 0%, 0.4% (balance in nitrogen), 5% (balance in nitrogen) and 21% (room air) O_2. A correlation coefficient of (R^2) > 0.99 was achieved for each calibration.

Headspace samples of 3 ml were drawn from the containers using 5 ml gas-tight syringes (Valco Instruments Co., Texas, USA), once daily at approximately the same time of day (± 30 minutes) for 14 consecutive days. Samples were injected directly onto the column, which was unheated.

2.2.3 Statistical analysis

Statistical analysis was performed using R Statistical Software (v.3.6.1 R Core Team, 2019). Generalised linear mixed-effect models (GLMM) using a beta family distribution with a logit link were used to assess CO_2 and O_2 gas levels against temperature, day, season, moisture and loading factor using maximum likelihood estimation (*glmmTMB* package, v.0.2.3 Brooks et al., 2017). A random group intercept was applied to log identity (ID) nested within the log group, to account for log section relatedness. The most parsimonious model was selected using leave-one-out cross-validation (Barr et al., 2013), in conjunction with visual inspection of model residual values versus fitted values using the *DHARMa* package (v.0.2.4 Hartig, 2019). Spearman correlation coefficient (r_s) was used to assess the monotonic relationship between CO_2 increase and O_2 decrease pooled across all seasons, as well as increase/decrease by time. Estimated marginal means were used for post-hoc data analysis of the interactions between the independent variables, season, temperature and time, using the *emmeans* package (v.1.4.1 Lenth et al. 2019). Local regression curves (LOESS), and raw, description inference (RDI) plots were used to display the data. RDI plots in this study show the spread of the raw data, the mean as the descriptive statistic, and Highest Density Interval (HDI) at 95% as the inferential statistics (Phillips, 2017).

2. 3 Results

Respiration of radiata pine logs (236.4 ± 1.6 mm average diameter, 205.9 ± 2.0 mm average height, n = 120) stored in airtight containers rapidly changed the atmospheric conditions within the container headspace. The increase in CO_2 production was variable depending on the temperature, ranging between 19 and 56% by the end of the 14-day experiment (Fig. 2). O_2 concentration rapidly decreased concomitantly with CO_2 increase, reaching hypoxic levels by day 2 and < 4% O_2 recorded for each container by day 14 (Fig. 2). An increase in CO_2 was highly negatively correlated with O_2 decrease over the experimental period (R = -0.83, P < 2.2e-16). There were significant seasonal variations in the rate of change and concentration of CO_2 and O_2 (Table 2).

Table 2. Parameter estimates from the generalised linear mixed-effect model for the variables affecting carbon dioxide (CO_2) production and oxygen (O_2) depletion by radiata pines (*Pinus radiata* D.Don) logs in airtight containers. Estimate represents the estimated regression coefficient ($\hat{\beta}$) for the population. Standard error is denoted as SE, and the z-value is the Wald z statistic.

	Carbon dioxide			**Oxygen**		
Parameters	***Estimate***	***SE***	***z-value***	***Estimate***	***SE***	***z-value***
Intercept	0.01	0.32	-16.36***	0.48	0.30	-2.44*****
15°C	1.08	0.20	0.36	1.30	0.13	2.08*****
20°C	1.79	0.19	3.06**	0.96	0.12	-0.32
†Day	2.134	0.25	-5.09***	0.76	0.09	-3.21******
Summer	0.283	0.26	-2.46***	1.42	0.14	2.58******
Autumn	0.526	0.24	-3.03*	1.56	0.15	2.87******
Winter	0.483	0.00	1.64**	1.39	0.14	2.39*****
Moisture	1.002	0.01	4.45	1.00	0.00	-1.07
Loading factor	1.022	0.25	-5.09*******	0.98	0.01	-3.85*******

P* at α = 0.05. * *P* < *0.05* ** *P* < *0.01* *** *P* < *0.001. † Day 2 presented in graph, days 2-14 all significant at *P* < *0.001*.

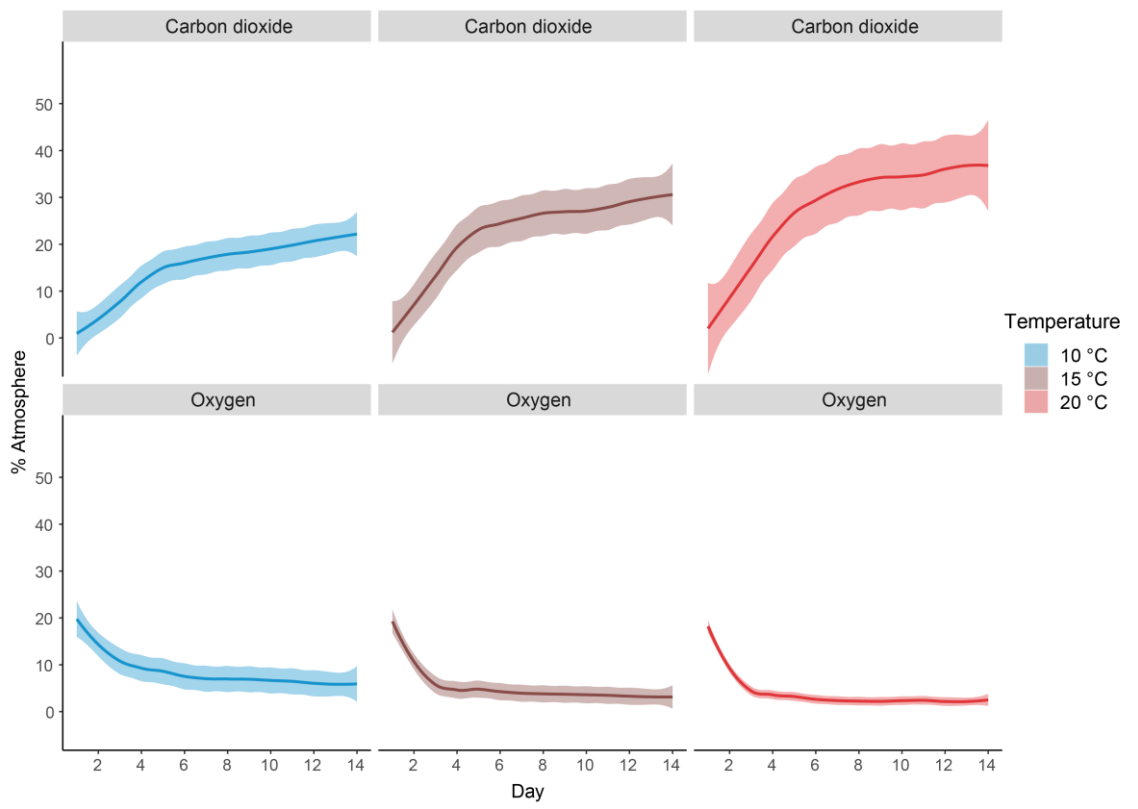

Fig. 2 Carbon dioxide (CO_2) increase and oxygen (O_2) decrease as a percentage of the atmosphere containing radiata pine (*Pinus radiata* D.Don) logs over 14 days at three different temperatures, which were representative of potential temperatures in a ship's hold. LOESS fitted lines represent the average CO_2 and O_2 for pine logs for all seasons combined (n = 120), with coloured areas showing 95% confidence intervals (CI).

2.3.1 Temperature effects

The higher temperature of 20°C had a significant positive effect on CO_2 production ($P < 0.01$, 95% CI = 1.23, 2.61). Conversely, temperature increase had a significant negative effect on O_2 reduction ($P < 0.1$, 95% CI = 1.02, 1.66) across all replicates (Table 2). The average (± SEM) CO_2 produced at 15°C (22.2 ± 0.5%) and 20°C (27.3 ± 0.7%) was significantly higher than for 10°C (15.1 ± 0.4%) across all replicates at the end of the 14-day period (Fig. 3). Similarly, O_2 concentrations at 15°C (5.5 ± 0.2%) and 20°C (4.2 ± 0.2%) were significantly lower when compared to 10°C (8.7 ± 0.2%) (Fig. 3).

Oxygen within the atmosphere rapidly decreased with increasing temperature, with an average daily decrease of -8% for 10°C, -10% for 15 and -11% for 20°C. The O_2 depletion from the logs harvested in summer showed the most rapid decrease per day at 20°C (-13%), the slowest rate of decrease was for autumn at 10°C (-1%).

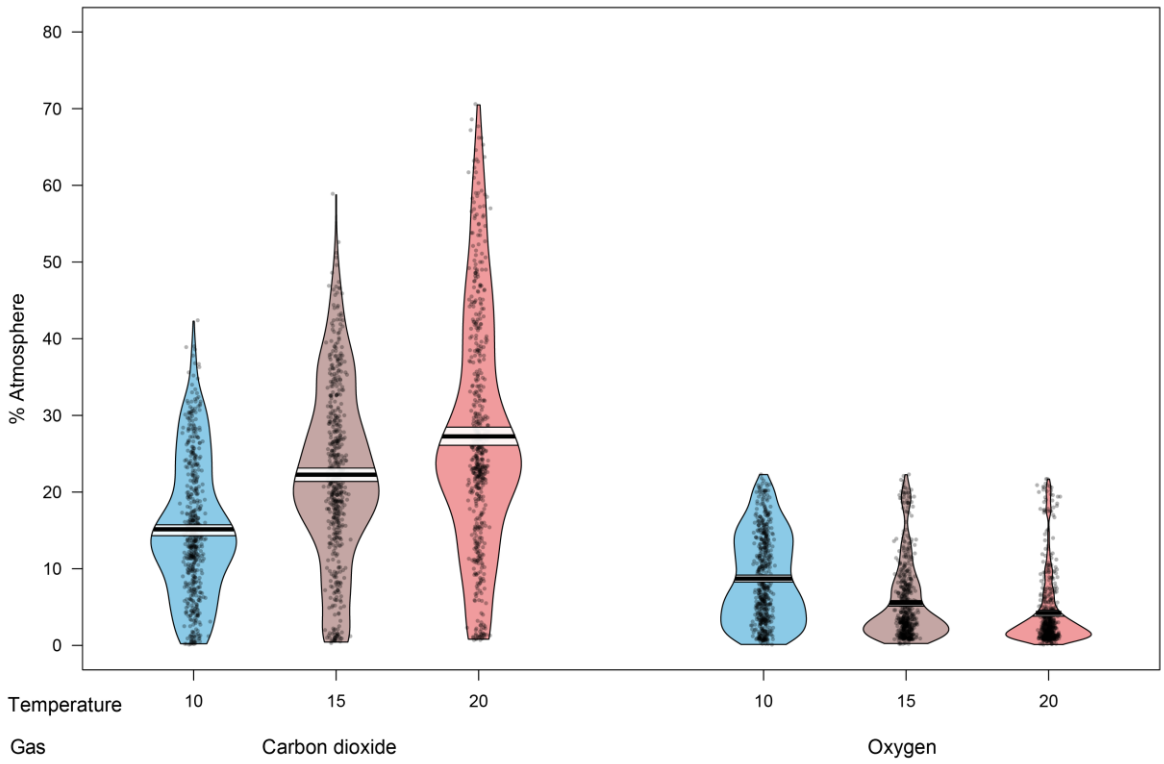

Fig. 3 Concentration of carbon dioxide (CO_2) and oxygen (O_2) in airtight containers housing radiata pine (*Pinus radiata* D.Don) logs (n = 30). Black dots represent raw data points, while violin shapes represent data probability density for each treatment. The white bands indicate 95% highest density intervals, and the black horizontal lines are the arithmetic mean for each treatment.

2.3.2 Time effects

Time from experiment initiation correlated with an increase in CO_2 from logs across all three temperatures (r_s = 0.64, P < 2.2e-16) and seasons. Consistent with this, an increase in time was significantly correlated with a negative effect on O_2 (r_s = -0.48, P < 2.2e-16).

On day 5, the average CO_2 percentage within the atmosphere was 14.7 ± 2.6% at 10°C, 23.0 ± 4.0% at 15°C, and 26.3 ± 4.5% at 20°C (Fig. 4). By day 10, CO_2 levels within the atmosphere had increased on average between 17.6 to 32.6% for all three temperatures, and on the final day of the experiment, the CO_2 readings were: 22.1 ± 0.5% at 10°C, 30.7 ± 0.6% at 15°C and 36.6 ± 0.6% at 20°C (Fig. 4).

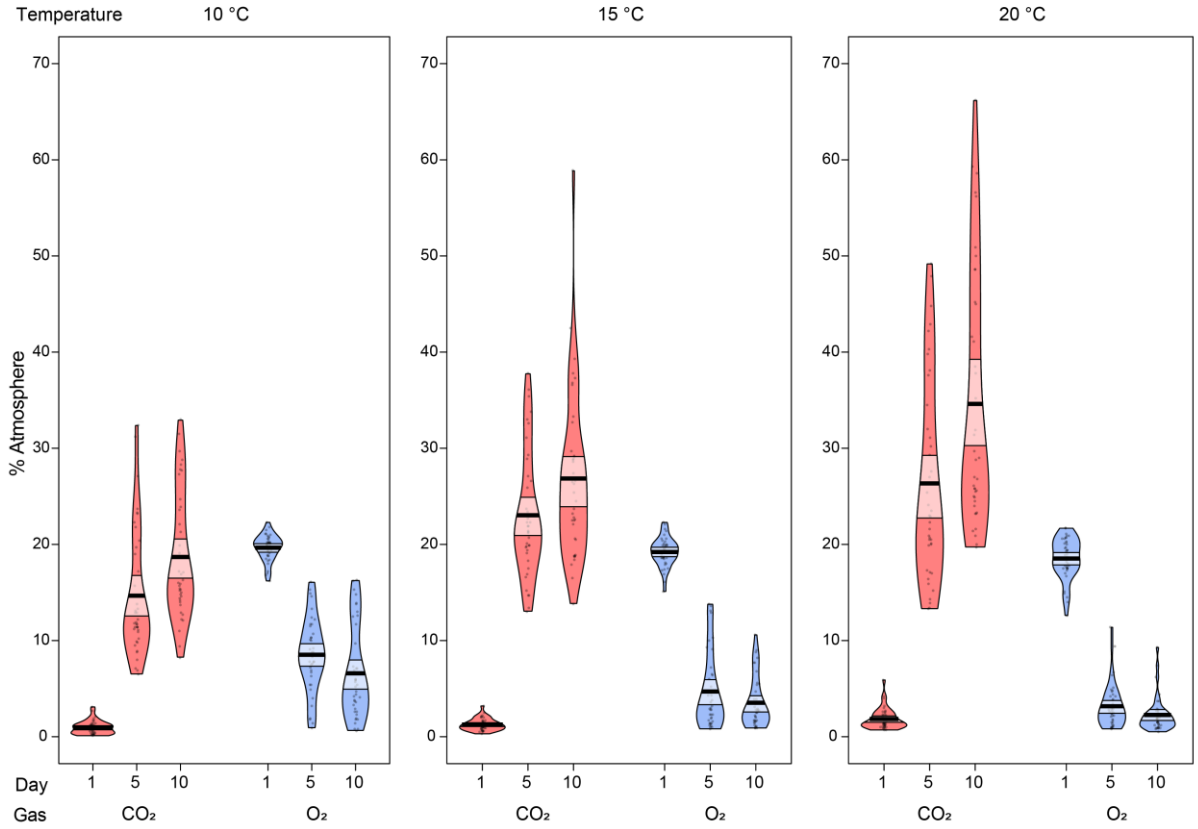

Fig. 4 Concentration of carbon dioxide (CO_2) and oxygen (O_2) in airtight containers housing radiata pine (*Pinus radiata* D.Don) logs (n = 30), with data shown for days 1, 5 and 10 after experiment initiation at 10, 15, and 20°C. Black dots represent raw data points, while violin shapes represent data probability density for each treatment. The white bands indicate 95% highest density intervals, and the black horizontal lines are the arithmetic mean for each treatment.

Oxygen depleted rapidly from the starting concentration of 20.9% within the container headspace, with concentrations falling below 10% by day 2. From day 5 onward, O_2 concentrations were ≤ 9% at 10°C, and ≤ 5% for both 15 and 20°C (Fig. 4). O_2 plateaued at ~ 5% from day 5 onwards at 15 and 20°C (Fig. 4). In comparison, at 10°C concentrations were twice this (~ 10%) by day 10 of the experiment.

2.3.3 Seasonal effects

The production of CO_2 from radiata pine logs was significantly different between all seasons except between spring and winter (Fig. 5). Autumn was highly significantly different compared to the other seasons at 20°C ($P < 0.001$) with an average of 40.5 ± 5.0% CO_2 by day 14, when compared to spring (22.0 ± 2.2%), summer (19.6 ± 2.0%) and winter (27.0 ± 2.0%). There were no significant differences between spring and winter for any of the temperatures for CO_2. For O_2, there was a significant seasonal effect between all the seasons at 10°C, except between spring and winter, which both had the least O_2 by day 14 (6.2 ± 1.3%, 6.6 ± 1.4%, respectively) as compared to the other seasons.

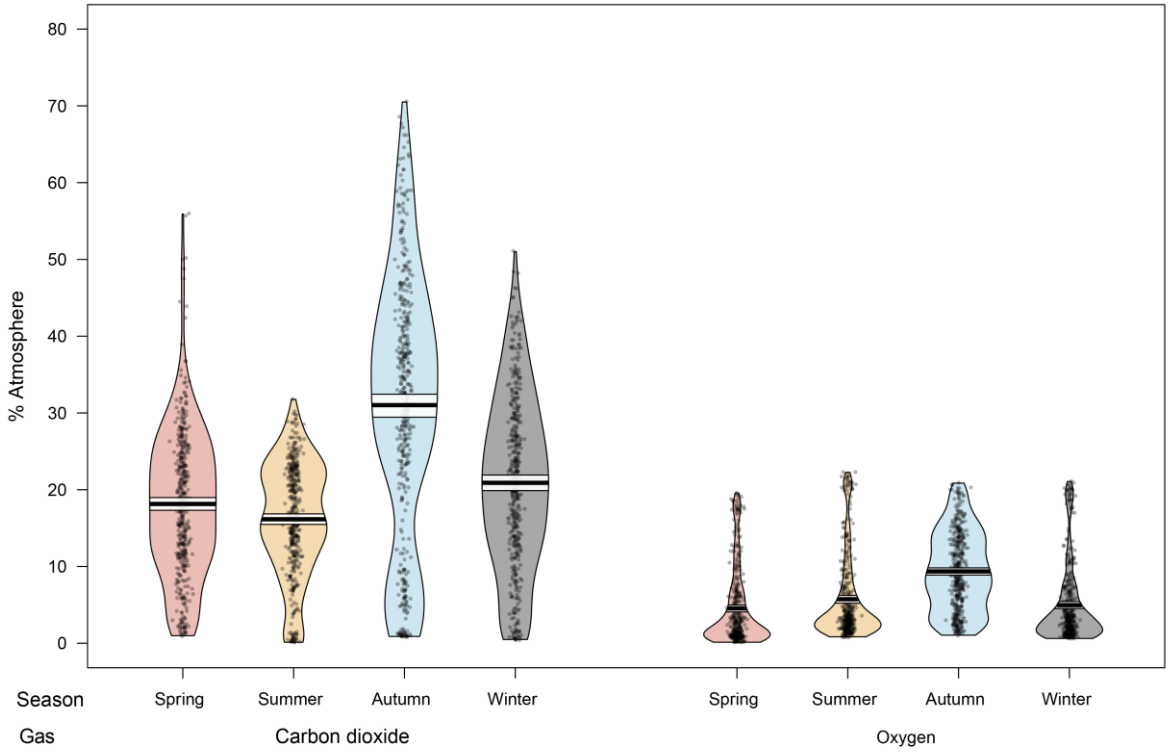

Fig. 5 Concentration of carbon dioxide (CO_2) and oxygen (O_2) in airtight containers housing radiata pine (*Pinus radiata* D.Don) logs (n = 30) for spring, summer, autumn and winter. Black dots represent raw data points, violins represent data probability density for each treatment. White band shows 95% highest density intervals, and the black horizontal lines are the arithmetic mean for each treatment.

2.3.4 Volume and moisture

The volume of the log within the container was correlated with a positive increase in CO_2 in the headspace atmosphere (GLMM model: $P < 0.001$, 95% CI = 1.01, 1.03); Table 2). However, Spearman regression indicated only a weak correlation between log volume and CO_2 increase ($r_s = 0.28$, $P <$ 2.2e=16). Moisture content of logs did not have any appreciable effect on CO_2 or O_2 (Table 2).

2.4 Discussion

Radiata pine logs within airtight containers drastically and rapidly modified the headspace atmosphere over the 14-day experimental period. Temperature had a measurable effect on CO_2 production by logs. An increase in temperature strongly correlated with an increase in CO_2, accompanied by a concurrent decrease in atmospheric O_2. The length of time that cut pine logs were held within the container was also a strong predictor of CO_2 production and O_2 reduction. There were seasonal effects associated with the amount of CO_2 production/O_2 reduction; autumn had higher levels of CO_2 compared to spring, summer and winter; while O_2 levels were lower in spring and summer than in other seasons. Moisture content and loading factor had no measurable impact on CO_2/O_2 levels.

Storage of unprocessed pine logs in an enclosed space rapidly led to a hypoxic/hypercapnic atmosphere, which can have important consequences for metabolism of the logs and associated biota. As early as day 2 for the warmer temperatures and by day 5 for all temperatures and seasons until the end of the experimental period, the gas levels reached are considered to be severely hypoxic/hypercapnic; O_2 concentration was extremely low ($\leq$ 2%) and CO_2 concentrations were extremely high (peaking at 54% in autumn at 20℃). Levels of CO_2/O_2 had more or less levelled out by the end of the experiment. CO_2 and O_2 production by the pine logs were similar to those reported by several other biological products' storage studies (e.g. Chidananda et al., 2014; Feng et al., 2015; Ochandio et al., 2017) and wood pellet transport reports (e.g. Svedberg et al., 2009; Tumuluru et al., 2013).

Oxygen shortage is likely to reduce mitochondrial metabolism in the logs, leading to substantial changes in their carbon and nitrogen metabolism (Planchet et al., 2017). Furthermore, hypoxic/hypercapnic conditions are likely to affect the metabolism of insects living in or on the logs (Cao et al., 2019). Hypercapnic environments have been shown to increase metabolism in some insects (Harrison et al., 2010) and reduce metabolism in others (Mitcham et al., 2006).

Insects also have varying levels of tolerance to hypoxia (Hoback & Stanley, 2001), although low O_2 tends to reduce insect metabolism (Harrison et al., 2018). Controlled atmosphere (low O_2) disinfestation is used to control some insect pests (Ahn et al., 2013); however, we think it unlikely that the modified atmosphere caused by pine logs will lead to significant insect mortality. Short-term exposure to high CO_2 concentrations generally does not cause insect mortality, but long-term exposure to > 40% CO_2 over several days might cause mortality in some insects (Boyer et al., 2012). High CO_2 concentration generally has a larger effect on insect mortality than low O_2 concentration because trehalose protects insect cells from low-O_2 stress (Cao et al., 2019). The net effect of hypoxic and hypercapnic conditions is likely to be complex, and dependant on the taxa involved.

An increase in temperature is associated with faster wood respiration, as well as faster microbiological respiration and biomass decomposition (Svedberg et al., 2009). This probably accounts for the strong correlation between increasing temperature and CO_2 produced by the cut radiata logs within the airtight containers in our study. CO_2 levels at 20°C were more than 55% higher than at 10°C. Higher temperatures can also cause autoxidation of fatty acids in wood products, which can lead to an increase in CO_2 production; while temperatures < 20°C seem to induce less production of volatile organic compounds compared to that at higher temperatures (Tumuluru et al., 2013). Temperature increase was found to be the dominant factor in CO_2 emissions from wood pellets (Kuang et al., 2009). We observed the same monotonic increase of CO_2 and decrease in O_2 in this study, with temperature being a significant predictor of CO_2 production.

There were also seasonal differences in respiration by cut radiata pine logs. The exception was that we detected no difference in respiration between spring and winter at all three temperatures. The fastest growing seasons for radiata pine in New Zealand coincide with the higher temperatures and sunshine hours of spring and summer (García, 1999), hence we expected that logs harvested during these two seasons would have higher respiration rates than those harvested in autumn and winter. Surprisingly, we observed the opposite. Plant respiration changes dynamically throughout the seasons, and can be highly variable depending on the temperature the tree is acclimatised to, as well as dependant on the tree species (Atkin & Tjoelker, 2003). Respiration rates increase more dramatically in radiata pine when saplings are acclimatised and moved from a low temperature (≤ 10°C) to a higher temperature (e.g. 20°C), than from ambient temperatures (15 - 20°C) to higher temperatures (25°C) (Ow et al., 2008). This may be why the rate and amount of CO_2 produced in our experiments were higher for the

logs from the two cooler months than the logs from the two warmer months; the temperature change from growing site to experimental conditions was more pronounced for the autumn/winter logs than the spring/summer logs.

One of the purposes of quantifying the amount and rate of changing CO_2/O_2 was to enable us to pinpoint when the atmosphere becomes hypercapnic/hypoxic, as this is important for assessing potential impacts on insect biosecurity. Insect control techniques that rely on insect respiration, such as fumigants that require active respiratory uptake (e.g. PH_3; Nayak et al., 2020), or metabolic suppression (i.e. cold treatments; Boardman et al., 2015) would likely be less effective if the pine logs modify the atmosphere to the extent that it alters insect respiration. Such changes in respiration probably vary according to the insect's developmental stage; egg and pupal life stages are generally more tolerant to hypoxia (Cheng et al., 2012). Several studies have indicated that a reduction in O_2 can induce narcosis in insects, conferring some protection against respiratory fumigants (Chaudhry, 1997; Liu et al., 2013; Liu, 2011). In contrast, increasing levels of CO_2 can increase the efficacy of PH_3 (Kashi & Bond, 1975; Ren et al., 1994). Thus, we recommend further work to assess the influence of hypoxic/hypercapnic conditions on the efficacy of PH_3 to control forest insect pests during log transit. It is critical for forest biosecurity to determine the most effective treatment protocols and corresponding optimal atmospheric conditions to maximise insect control in unprocessed logs. Reducing the impacts of log respiration with improved ventilation or the addition of O_2 may be one way of achieving more optimal atmospheric conditions during transport, although this may not be feasible in practice.

2.5 Conclusion

Radiata pine logs induce hypoxic/hypercapnic atmospheric levels within days of airtight storage. There was strong support for our hypothesis that higher temperatures increased the rate and amount of CO_2 produced, as well as reducing O_2 at a faster rate than at lower temperatures. There was also evidence to support our hypothesis that season influences log respiration. However, this was not in the direction predicted, as rates were higher in autumn and winter than spring and summer. The amount and timing of changes in CO_2 and O_2 atmospheric levels induced by respiring radiata pine logs in enclosed space indicate that the efficacy of some insect control treatments is likely to be reduced. Further research is needed to counteract the impact of hypercapnic/hypoxic atmospheres on insect control efficacy.

Chapter 3 - Metabolic response to hypoxia and hyperoxia in an endophagous bark beetle, *Hylurgus ligniperda* (F.)

3.1 Introduction

Insects are a diverse group that inhabit a wide range of different environments. Some species have physiological adaptations that allow them to survive in extreme modified atmospheres (Hoback, 2012). One example is the microhabitat of endophagous (living within the host) insects such as borers, miners and gall forming insects (Hoback & Stanley, 2001). Although poorly understood, this environment is likely hypoxic (low O_2), and hypercapnic (high CO_2) compared to ambient atmospheric levels (Pincebourde & Casas, 2016). It is thought that tunnel-boring insects are exposed to severe hypoxia (< 5% O_2) as gas diffusion in the cambium is generally limited (Pincebourde & Casas, 2016). Gas exchange patterns and atmospheric tolerances in endophagous species are largely unknown (Pincebourde & Casas, 2016). However, it is likely many of these species have adaptations to their metabolism and respiration that allow them to survive in low O_2/high CO_2 environments (Hoback & Stanley, 2001). This would be consistent with adaptations found in dung beetles that have also evolved in a hypoxic environment; these beetles occupy fresh dung with O_2 levels as low as 1-2% for extended periods of time and they can regulate their O_2 between 2-20.9% (Holter & Spangenberg, 1997).

The amount of O_2 required by an insect is dependent on its internal partial pressure of oxygen (P_{O_2}), which can vary significantly among species and between different life stages. Insects deploy a range of mechanisms to regulate their internal P_{O_2}. (Chown & Nicolson, 2004). Some species exhibit periods of intermittent gas exchange, where spiracles open, close and 'flutter' in a cyclic pattern, termed discontinuous gas exchange (DGC) (Chown et al., 2006). Others show different patterns, with regular bursts of gas release (cyclic), or continuous gas exchange (continuous), where there is no discernible pattern (Terblanche & Woods, 2018). Short term exposure to mildly hypoxic atmospheres generally is not detrimental for most insects (> 10 kPa) (Harrison et al., 2006). Severe hypoxia (< 2%) on the other hand can have lethal effects on insects. However, the detrimental impacts can be highly variable depending on the species, the life stage at exposure, and the ecology of the species (Hoback, 2012; Harrison et al., 2018). When insects are subjected to a reduction in environmental P_{O_2} to below ambient levels (20.9

kPa), they can compensate by increasing ventilation (via increased spiracle opening) and by lowering their demand for O_2 through minimising activity (Harrison et al., 2006; Harrison et al., 2018). Under circumstances where the insects internal P_{O_2} need exceeds what is available in the environment despite compensation attempts, other processes are utilised (Harrison et al., 2018). A full or partial switch to anaerobic metabolic pathways and severe metabolic suppression have been observed as a response to hypoxia (Schmitz & Harrison, 2004; Woods & Lane, 2016). This allows some insects to survive several weeks even under anoxic conditions (0% O_2) (Hoback, 2012).

Insects that are adapted to hypoxic environments have shown cross-tolerance to other abiotic stressors (Boardman et al., 2011; Boardman et al., 2015). For instance, insects that are acclimated to hypoxic conditions prior to cold exposure are able to withstand low temperatures for longer periods of time than those that are not pre-exposed (Boardman et al., 2015). Similarly, hypoxia-tolerant insects are more resistant to control measures such as low oxygen (O_2) controlled atmospheres (Mitcham et al., 2006). For example, irradiation under extreme hypoxia (< 1% O_2) is less effective than irradiation under normal atmospheric O_2 (20.9%) (Condon et al., 2017). Resistance to chemical controls, such as resistance to PH_3, also has been linked with tolerance to hypoxic conditions (Nayak et al., 2020). Insects across their life stages can also show differences in their tolerance to low O_2 environments (Belozerov, 2009; Woods & Lane, 2016). Generally, less mobile life stages such as pupae and eggs have been observed to have dramatically reduced metabolism compared to more mobile life stages, such as adults and larvae (Koštál, 2006; Hahn & Denlinger, 2011). Most pupae are in a state of dormancy in which direct developmental processes are arrested (Danks, 1987). It is thought that, with the selective suppression of principle growth processes, other processes such as stress-resistant mechanisms are increased (Denlinger et al., 2012; King & MacRae, 2015). This means that during dormant or quiescent phases (e.g. pupae), some insects are more tolerant to adverse conditions such as temperature extremes (Lester & Irwin, 2012) and hypoxia (Michaud et al., 2011). Pupal and egg life stages of some insects are more tolerant to various control methods (e.g. thermal disinfestation, Loganathan et al., 2011; ozone disinfestation, McDonough et al., 2011; chemical control Manivannan, 2015). Understanding the respiration of a species across its life stages allows us to understand the physical limits of that species, and how this may affect control methods.

Bark beetles (Curculionidae: Scolytinae) are endophagous herbivores for nearly their entire life cycle; only adults leave the cambium, in order to mate and locate new hosts (Raffa et al., 2015). Although bark beetles have evolved to survive the low O_2 environment within a tree, little is known about their specific physiological adaptations to hypoxic conditions. This is particularly important to understand for bark beetles because they are considered important forest pests (Kirkendall et al., 2015). They can aggregate in large numbers, killing large tracts of forest, and are easily and frequently transported to novel areas through the trade in wood and plant products (Sweeney et al., 2019; Javal et al., 2019). Significantly detrimental examples are species within the *Dendroctonus* and *Ips* genera, which encompass some of the most notorious conifer-killing species (Six & Bracewell, 2015). The European bark beetle, *Hylurgus ligniperda* (F.) is a widespread invasive pest in pine forests and plantations in many regions, including Australia, South Africa, North and South America and New Zealand (Reay & Walsh, 2001; Faccoli et al., 2020). Despite an increase in awareness of the potential damage and the ease of movement associated with invasive bark beetles (Brockerhoff & Liebhold, 2017), they are still frequently intercepted in wood and plant products (Faccoli et al., 2020). Effective disinfestation of these types of products from bark beetles is essential for halting their continued spread and maintaining market access (Hlásny et al., 2019). Disinfestation may be improved if we can identify how the physiology of bark beetles responds to different O_2 levels and whether this is likely to alter the efficacy of control tools.

Our aim in this study was to assess the respiration physiology of bark beetles, using *H. ligniperda* as our model. We measured the respiration rates and the critical oxygen threshold limit (P_{crit}) of three life stages of *H. ligniperda.* We used stop-flow respirometry to ascertain the respiration of *H. ligniperda* larvae, pupae, and adults under three O_2 treatments: hypoxia (2 kPa), normoxia (20.9 kPa), and hyperoxia (40 kPa). We included two temperatures, 10°C and 20°C, and two humidity treatments – dry (0% RH) and wet (95% RH). We included a dry humidity treatment as water conservation has been posited as a potential trigger for DGC (see hygric hypothesis; Chown et al., 2006). We then used flow-through respirometry to assess how tolerant the three *H. ligniperda* life stages were to low O_2 environments, using a stepwise reduction in O_2 (21-0.5 kPa) in order to establish the lowest level of O_2 this species could tolerate before metabolic shut-down occurred.

We predicted that all life stages of *H. ligniperda* would be tolerant to severe hypoxia (≤ 2% atmospheric O_2) and that respiration would vary with life stage. We hypothesised that pupae would be the most tolerant to hypoxia because this life stage is likely already in a state of

dormancy. We also expected that metabolic rates of all life stages would decrease as temperature, humidity and atmospheric O_2 decreased.

3.2 Methods

3.2.1 Insects

We obtained *H. ligniperda* larvae, pupae and adults from a laboratory colony at Plant and Food Research, Auckland, New Zealand during May 2018. This colony was established in 2012 with field-collected insects from the Central North Island and Canterbury Region, New Zealand (Clare & George, 2016). The insect colony at Plant and Food Research is kept in 24-hour darkness, ~ 60% relative humidity at 20°C and maintained on an artificial insect diet consisting predominantly of radiata pine (*Pinus radiata*) sawdust, wheatgerm, cellulose, agar and water (See 'diet 5' in Barrington et al., 2015). When we received the insects, we maintained them on the same artificial diet and stored them at 10°C to slow metamorphosis. The average (± SEM) age of each life stage in days from oviposition was 29.7 ± 0.4 for larvae, 40.3 ± 0.3 for pupae, and 79.8 ± 0.7 for adults.

Twenty-four hours before use in respirometry experiments we removed the insects from the artificial diet and placed them in a petri dish lined with moist tissue paper. We determined the sex of adult *H. ligniperda* prior to experimentation by gently holding the insect between the thumb and forefinger while listening for the 'chirping' sound produced by the male (Clare & George, 2016). If we did not hear chirping after 20-30 seconds, the insect was deemed female (Clare & George, 2016).

3.2.2 Atmosphere modification

Oxygen (O_2) levels were modified using a ROXY-4 (Sable Systems International) universal gas regulator, and humidity was modified using a custom dew-point regulator (Lighton, 2008). Firstly, room air was drawn through a layered Drierite-Ascarite-Driertie column to scrub CO_2 and water vapour. This dry, CO_2-free air was drawn into a 22 L carboy, where the different O_2 levels were mixed. For normoxia (20.9 kPa) the scrubbed room air was used. For the hypoxia (2 kPa), additional nitrogen (BOC Gases New Zealand Ltd, Auckland) was periodically supplied to the carboy via the ROXY-4 (SSI) to maintain an O_2 level of 2 kPa. For hyperoxic (40 kPa) atmospheres additional O_2 (BOC Gases New Zealand Ltd, Auckland) was similarly maintained and moderated by the ROXY-4 (SSI). O_2 levels within the carboy were monitored

using a ROXY-4 O_2 fuel cell sensor (SSI). As an additional measure a second fuel cell was placed at the end of the reference line of the respirometry setup to verify O_2 set points. For the dry (0% RH) atmospheres air was drawn directly from the carboy, and for humidified atmospheres (90% RH) the carboy air was re-humidified by bubbling it through a custom dew-point generator. A relative humidity of 90.1 ± 0.5% was achieved by bubbling warm (20°C ± 5) air through water at 8.6 ± 0.1°C (for recordings at 10°C) or water at 18.1 ± 0.2°C (for recordings at 20°C) (Lighton, 2008).

3.2.3 Respirometry set-up: stop-flow

We used stop-flow respirometry with automatic bolus integration to measure the rates of CO_2 emission ($\dot{V}CO_2$) from *H. ligniperda* beetles at each life stage in response to temperature, humidity and O_2 treatment (as per Lighton, 2008). We measured CO_2 emission once, with one of the following combinations of treatments: 10 or 20°C, humidity as either wet (90% RH) or dry (0% RH), under one of three O_2 treatments, 2, 20.9 or 40 kPa.

We used Sable Systems International (SSI) stop-flow and flow-through respirometry systems with a LI-7000 infrared gas analyser. Dry, CO_2-free air was flushed at 200 mL min^{-1} sequentially through each chamber for 10 min using the multiplexor (RM8, SSI), and then each chamber was sealed for 50 minutes. After 50 minutes, the accumulated CO_2 within the chambers was sequentially flushed through a column of regenerated Drierite (White et al., 2006) before entering the LI-7000 analyser (plumbed in differential mode) at 200 mL min^{-1} against a baseline stream of dry, CO_2-free air. A blank chamber was included in each respirometry recording as a control chamber to account for any CO_2 that may have entered the solenoid valves or tubing. The temperature was thermoelectrically controlled within the temperature-controlled cabinet using a peltier control device (PELT-4; SSI), and insects were kept in the dark during the recordings.

Insects (n = 4 per run) were randomly selected and measured sequentially during the 85-minute recording. We weighed, then placed, insects in a custom-made chamber, which consisted of 3 cm long sections of Bev-A-Line tubing, enclosed by connectors (Fig. 1) (Miniature Quick-Disconnect Fitting, Cole-Parmer, Canada). A small (2 mm) piece of fine wire mesh (approx. 0.5 mm x 0.5 mm) was used to cover the connector openings to stop the insects from crawling into the tubing. The order of insect life stages was randomised between chambers, and the order of the treatment conditions was randomised between days. Each recording was drift-corrected

to baseline recordings of a stream of dry, CO_2-free air running through an empty chamber at the start and end of each run.

We excluded several pupae (n = 9) in dry, 10°C treatments because the volumes of CO_2 they produced was indistinguishable from the empty chamber.

Fig. 1 *Hylurgus ligniperda* (F.) larva (top chamber), and adults (middle and bottom chamber) within custom-made Bev-A-Line tube chambers.

We calculated the volume of CO_2 following Lighton (2008):

$$\dot{V}CO_2 = FR_i(F_eCO_2\text{-}F_iCO2)$$

where $\dot{V}CO_2$ is the rate of carbon dioxide (in μL min^{-1}), F_eCO_2 is the excurrent fractional concentration of CO_2, F_iCO_2 is the incurrent fractional concentration of CO_2, and FR_i is the incurrent flow rate. The CO_2 trace for each insect chamber flushing period was integrated against the baseline CO_2-free air, which enabled the volume of CO_2 produced by the insects to be calculated. The CO_2 volume from the flushed empty chamber was then subtracted from each

insect chamber's CO_2 volume during each run. The volume of each insect chamber was then divided by the time that the chambers were sealed to obtain $\dot{V}CO_2$ in μL min^{-1}.

3.2.4 Respirometry set-up: flow-through

To establish the critical oxygen threshold (P_{crit}) for *H ligniperda* larvae, pupae and adults, we used the same respirometry system except for a minor modification: a small column of regenerated Drierite was placed after the mixing carboy and before the respirometry setup to ensure the air was free of moisture. Atmospheric O_2 was decreased incrementally through ten, 10-minute steps from 21 kPa - 0.5 kPa (21, 16, 13, 9, 7, 5, 3, 2, 1, and 0.5 kPa) using the Roxy-4, with only the last 5 minutes of each of the 10-minute steps recorded. Dry, CO_2-free air was drawn at 80 mL min^{-1} through a 10 cm^3 glass chamber containing an individual beetle or a blank chamber. The insects were kept in darkness during the recordings in a temperature-controlled cabinet set to 20°C (PELT-4, SSI). We recorded insect activity using an AD-1 infrared activity detector (SSI). CO_2 in the excurrent air from the insects within the chambers was measured using the LI-7000 in a differential configuration. Baseline drift was corrected as per stop-flow respirometry, and the rates of CO_2 were calculated as follows according to Lighton (2008):

$$\dot{V}CO_2 = FR_i(F_eCO_2\text{-}F_iCO2)$$

where $\dot{V}CO_2$ is the rate of carbon dioxide (in μL min^{-1}), F_eCO_2 is the excurrent fractional concentration of CO_2, F_iCO_2 is the incurrent fractional concentration of CO_2, and FR_i is the incurrent flow rate.

To determine an index of the activity of beetles during flow-through respirometry we took the absolute difference sum (actADS) of the AD1 activity channel. The units of the activity channel are arbitrary; deflections from baseline indicate periods of movement. However, the slope of the actADS trace approaches zero during periods of no activity. For the whole trace we sampled a 300-second interval with the lowest actADS slope (using the nadir function in Expedata; SSI; V.1.8.5) and calculated the mean and standard deviation of the slope actADS. We considered any data points with an actADS slope above ten standard deviations to be periods of activity. A 60-second region within each level of atmospheric O_2 was selected with the least duration of the activity. The volume of $\dot{V}CO_2$ was then averaged over the selected 60-second region as an estimate of $\dot{V}CO_2$. Following (Wood, 2018) we fitted a Michaelis-Menten function to $\dot{V}CO_2$:

$$\dot{V}CO_2 = (\dot{V}CO_{2,max} \times P_{O_2}) / (K_m + P_{O_2})$$

where $\dot{V}CO_2$ is the rate of carbon dioxide (in μL min^{-1} g^{-1}), $\dot{V}CO_{2,\,max}$ is the maximum CO_2 emission (i.e. $\dot{V}CO_2$ during normoxia), P_{O_2} is the partial pressure of oxygen, and K_m is the P_{O_2} at which $\dot{V}CO_2$ is 50% of maximum (i.e. a proxy for the affinity of the insect for O_2). To estimate P_{crit} we followed Marshall et al. (2013). Briefly, to account for the variation in absolute slope between individuals $\dot{V}CO_2$ data for each individual were normalized, such that $\dot{V}CO_{2,max} = 1$ and $\dot{V}CO_{2,min} = 0$. We then took the derivative of this function and solved for the P_{O_2} at a slope of 0.065 (a non-zero threshold defining when the slope approaches zero; Marshall et al. 2013). This value was taken as the P_{crit}. At the P_{crit} insects generally open spiracles and maximize ventilation (Greenlee & Harrison, 2004). Under these conditions, the tracheal system's maximal conductance for O_2 delivery (G_{max} from air to mitochondria assuming tracheolar fluid is removed; Greenlee & Harrison, 2004; Lease et al., 2012) can be calculated. Firstly, $\dot{V}O_2$ from $\dot{V}CO_2$ assuming an RQ of 0.8 (Lighton, 2008):

$$\dot{V}O_2 = \dot{V}CO_2 / RQ$$

Then G_{max}:

$$G_{max} = (\dot{V}O_2 / P_{O_2})$$

Time moving at each P_{O_2} was reported as the number of seconds (out of 300) when the slope of the activity ADS was ten standard deviations greater than a period of zero activity (i.e. 10 × SD of the instrument noise of the AD1).

3.2.5 Statistical analysis

All statistical analyses were performed using R statistical software (v.3.5.3, R Core Team, 2019). We used quartile to quartile plots as well as the Shapiro-Wilks test for normality to evaluate respiration rates from the stop-flow and flow-through experiments. Insect $\dot{V}CO_2$ data were not normally distributed in the stop-flow experiment, as variance increased with the mean. Hence a generalised linear model (GLM) with a gamma distribution and log link function was selected to compare mass-corrected $\dot{V}CO_2$ ($\dot{V}CO_2/M_b = \dot{V}CO_{2,ms}$) between life stages against the predictor variables of temperature, humidity and atmospheric O_2. We compared several models and selected the model with the lowest Akaike information criterion (AIC) (Burnham & Anderson, 2002), along with the most parsimonious residual plots (*DHARMa* package

v.0.2.4 Hartig, 2019). As humidity had no significant effect on $\dot{V}CO_2$ output (GLM model: P = 0.6), the humidity covariate was omitted in the better-fitting model. For post-hoc pairwise comparisons, estimated marginal means were calculated using the *emmeans* package (v.1.4.1 Lenth et al. 2018). For the flow-through experiments, we compared P_{crit}, $\dot{V}CO_{2,max}$, K_m and G_{max} between life stages using analysis of variance (ANOVA) for each parameter separately, and then pairwise contrasts were calculated using Tukey's Range test.

3.3 Results

3.3.1 Gas exchange patterns of *Hylurgus ligniperda* at normoxia

All *Hylurgus ligniperda* life stages displayed a continuous gas exchange pattern under normoxia, and under the different treatment conditions (Fig. 2). At 10°C, the average (±SEM) larval $\dot{V}CO_2$ (in μL min^{-1}) was 2.29 ± 0.21, pupal $\dot{V}CO_2$ was 1.25 ± 0.13, and the adult $\dot{V}CO_2$ was 3.32 ± 0.43. Respiration significantly increased for all life stages at 20°C compared to 10°C (Fig. 3). At 20°C, the larval average $\dot{V}CO_2$ was 6.93 ± 0.6, the pupal average was 4.9 ± 0.36, and adult $\dot{V}CO_2$ was 10.37 ± 1.25. Pupae CO_2 emissions under normoxia were significantly lower than larvae and adults at 10°C, and significantly lower than adults but not larvae at 20°C (Fig. 3).

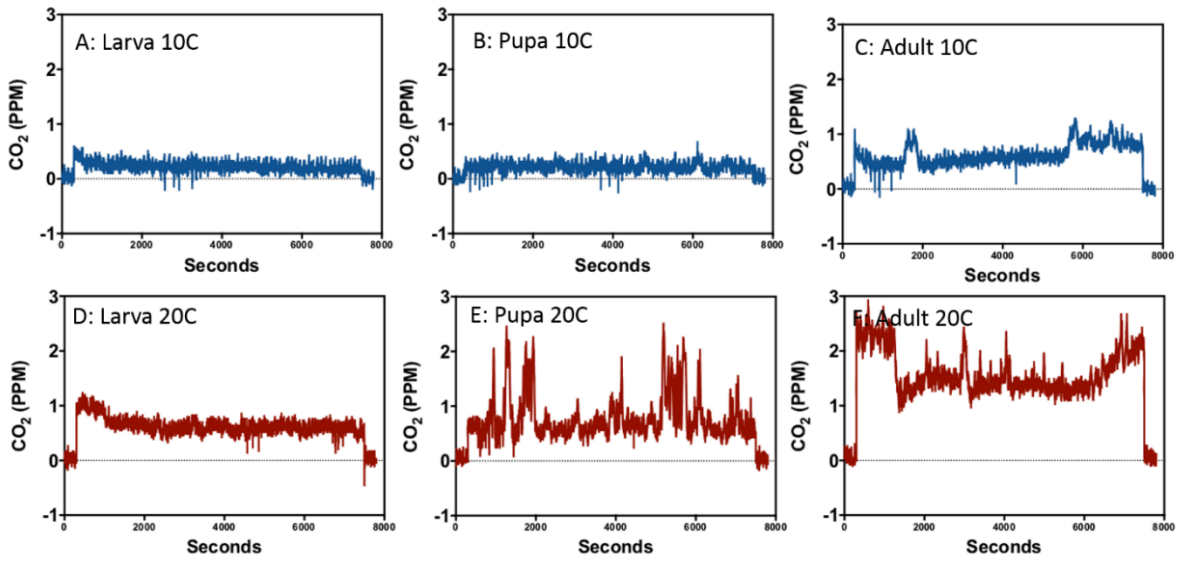

Fig. 2 Flow-through respirometry traces of CO_2 production in parts per million (ppm) from a single insect at each life stage of *Hylurgus ligniperda* (F.) at 10°C and 20°C. Graphic by Kurtis Fielding Turnbull, 2017, used with permission.

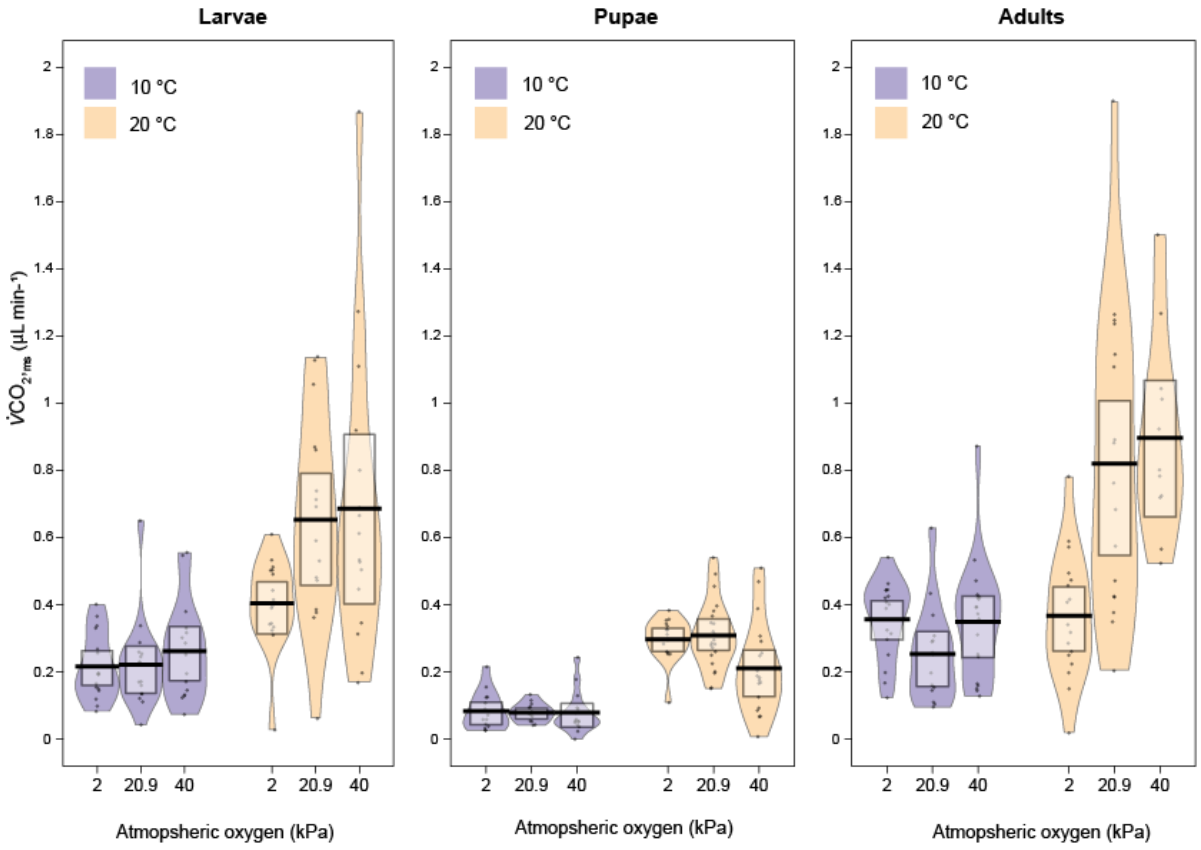

Fig. 3 Mass-corrected volume of CO_2 output (in µL min^{-1}) ($\dot{V}CO_{2,ms}$) for *Hylurgus ligniperda* (F.) larvae (n = 97), pupae (n = 100) and adults (n = 96) under three different O_2 atmospheric treatments (2, 20.9 and 40 kPa), and two temperature treatments (10 or 20°C). Violin shapes represent the data probability density for each treatment, raw data points are represented by the black dots. The black horizontal lines represent the arithmetic mean for each treatment with the lighter surrounding box representing 95% highest-density intervals for each data set.

3.3.2 Response to atmosphere and temperature by life stage

CO_2 emission by *H. ligniperda* was significantly different between the life stages in response to the varying O_2 and temperature treatments (Fig. 4). At 10°C under a hypoxic atmosphere (2 kPa) the respiration of larvae, pupae and adults were significantly different from one another. Pupae had the lowest average $\dot{V}CO_2$ (1.30 ± 0.22), followed by larvae (2.44 ± 0.25), and then adults (4.71 ± 0.37) in the 10°C hypoxic treatment. Under normoxia (20.9 kPa) and hyperoxia (40 kPa) at 10°C, larval and adult respiration did not differ significantly, however pupal respiration was significantly lower than the respiration of larvae and adults (Fig. 4, Table 1).

CO_2 emission by the insects at 20°C in hypoxia did not vary significantly between the life stages (Fig. 4). At this temperature, the only significant differences were between pupae and

adults at normoxia and hyperoxia, and between larvae and pupae at hyperoxia (Table 1; Fig. 4). O_2 atmosphere had no significant effect on pupal respiration rates at 10°C, with pupal $\dot{V}CO_2$ overall for 10°C varying by only ~ 0.05 between atmospheres (Fig. 4).

Temperature had a significant effect on the beetle's metabolism (GLM model: $P > 0.001$, Table 2), with respiration increasing concurrently with temperature for all life stages (Fig. 4). The individual mass of the insect also had a significant effect on their respiration ($P > 0.001$; Table 2).

Table 1. Pairwise comparison of the contrasts between life stages of *Hylurgus ligniperda* (F.) in response to the different partial pressures of O_2 (2, 20.9, and 40 kPa) in combination with the two temperature treatments (10 and 20°C) using estimated marginal means with Tukey adjusted *P* values (at $\alpha = 0.05$).

Partial pressure of O_2	***Life stage contrasts***	***Difference by temperature***	
		10°C	**20°C**
2%	Larva – pupa	**	ns
	Larva – adult	**	ns
	Pupae – adult	***	ns
20.9%	Larva – pupa	***	ns
	Larva – adult	ns	ns
	Pupae – adult	***	***
40%	Larva – pupa	***	***
	Larva – adult	ns	ns
	Pupae – adult	***	***

ns, not significant, * P < 0.05 ** P < 0.01 *** P < 0.001

Table 2. Model parameter estimates of mass-corrected CO_2 output by volume (in μL min^{-1}) ($\dot{V}CO_{2,ms}$) from *Hylurgus ligniperda* (F.) larvae, pupae and adults in contrast to predictor variables of atmospheric O_2 (2, 20.9 or 40 kPa), temperature (10 or 20°C), and insect mass in milligrams. Parameter estimates from generalised linear model (GLM) with a gamma distribution and a log link function showing estimate, standard error (SE), *t* value, and *P* values (at $\alpha = 0.05$) with Nagelkerke pseudo R^2 as an estimate of the goodness of fit.

Predictor variable	***Estimate***	***SE***	***t value***	***P***
Intercept	-1.59	0.29	-5.43	***
Mass (mg)	-0.07	0.01	-5.79	***
Temperature (C)	0.08	0.02	4.81	***
O_2 level	0.00	0.01	-0.29	ns
Life stage: Pupa	-1.30	0.38	-3.39	***
Life stage: Adult	1.18	0.37	3.21	**
Temperature x O_2 level	0.00	0.00	1.08	ns
Temperature x pupa	0.06	0.02	2.62	**
Temperature x adult	-0.06	0.02	-2.70	**
O_2 level x pupa	0.02	0.01	1.05	ns
O_2 level x adult	-0.02	0.01	-1.68	ns
Temperature x O_2 level x pupa	0.00	0.00	-1.99	*
Temperature x O_2 level x adult	0.00	0.00	2.08	*
Observations	239			
Nagelkerke R^2	0.68			
Deviance	83.8			
AIC	-303.959			

ns, not significant, * P < 0.05 ** P < 0.01 *** P < 0.001

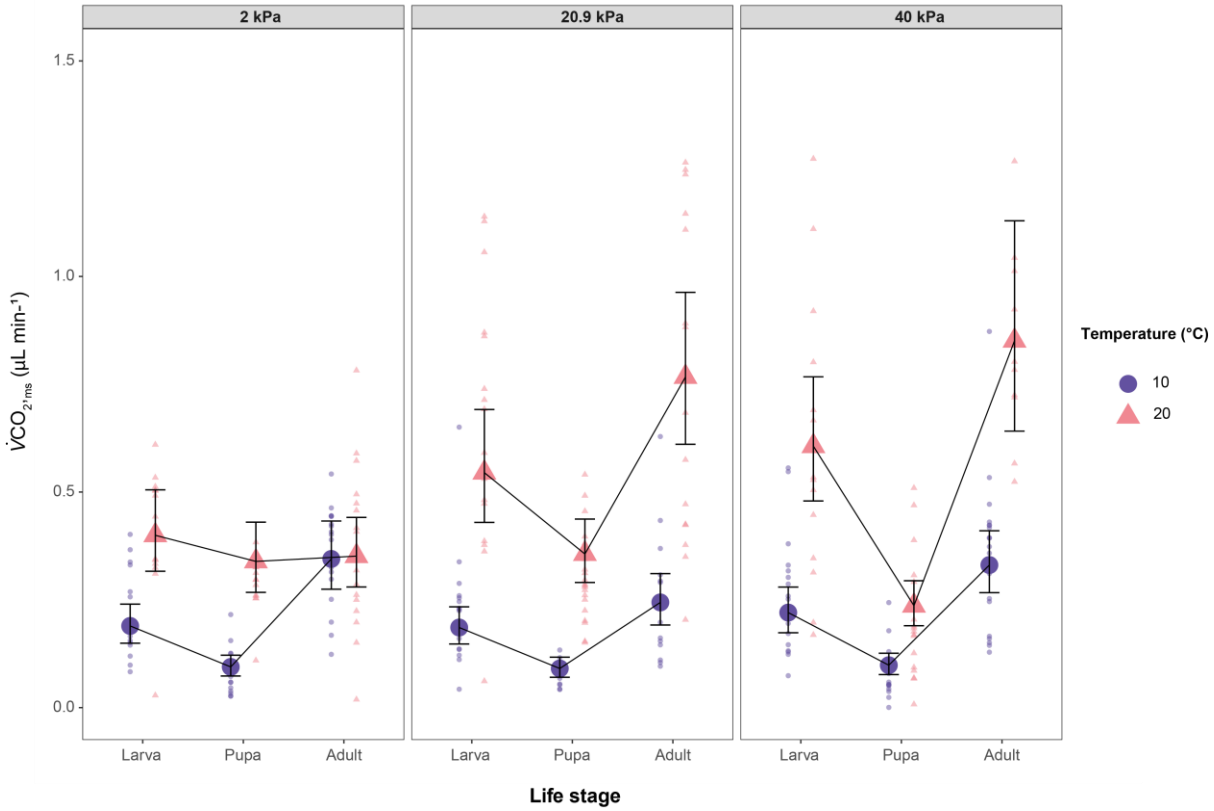

Fig. 4 Pairwise comparisons of carbon dioxide ($\dot{V}CO_{2,ms}$) emissions by *Hylurgus ligniperda* (F.) life stages at different concentrations of atmospheric O_2 (2, 20.9 and 40 kPa) calculated using estimated marginal means of the mass-corrected volume (in µL min^{-1}). Coloured shapes are the estimated marginal means and the error bars represent ± SEM.

3.3.3 Critical partial pressure of oxygen

Each life stage of *H. ligniperda* responded differently to a stepwise reduction in O_2 (Fig. 5). The critical threshold of oxygen (P_{crit}) was significantly different across the life stages ($F_{2,\ 17}$ = 4.89, P = 0.02) in the omnibus test. However, post hoc analysis revealed that the only pairwise significant difference was between larvae and pupae. Larvae had the highest average (± SEM) P_{crit} at 3.8 ± 0.01 kPa, followed by adults 2.8 ± 0.14 kPa, and then pupae with the lowest P_{crit} at 2.5 ± 0.01 kPa (Fig. 5 & 6A). The maximum conductance of the tracheal system for O_2 (G_{max}) and the partial pressure of O_2 in which consumption is half the maximal (K_m) were also significantly different among the life stages (G_{max}: $F_{2,\ 17}$ = 7.44, P = 0.02; K_m: $F_{2,\ 17}$ = 5.07, P > 0.005; Fig. 6C & 6D respectively). The maximum $\dot{V}CO_2$ emission at normoxia ($\dot{V}CO_{2,max}$) was not significantly different among the life stages (Fig. 6B).

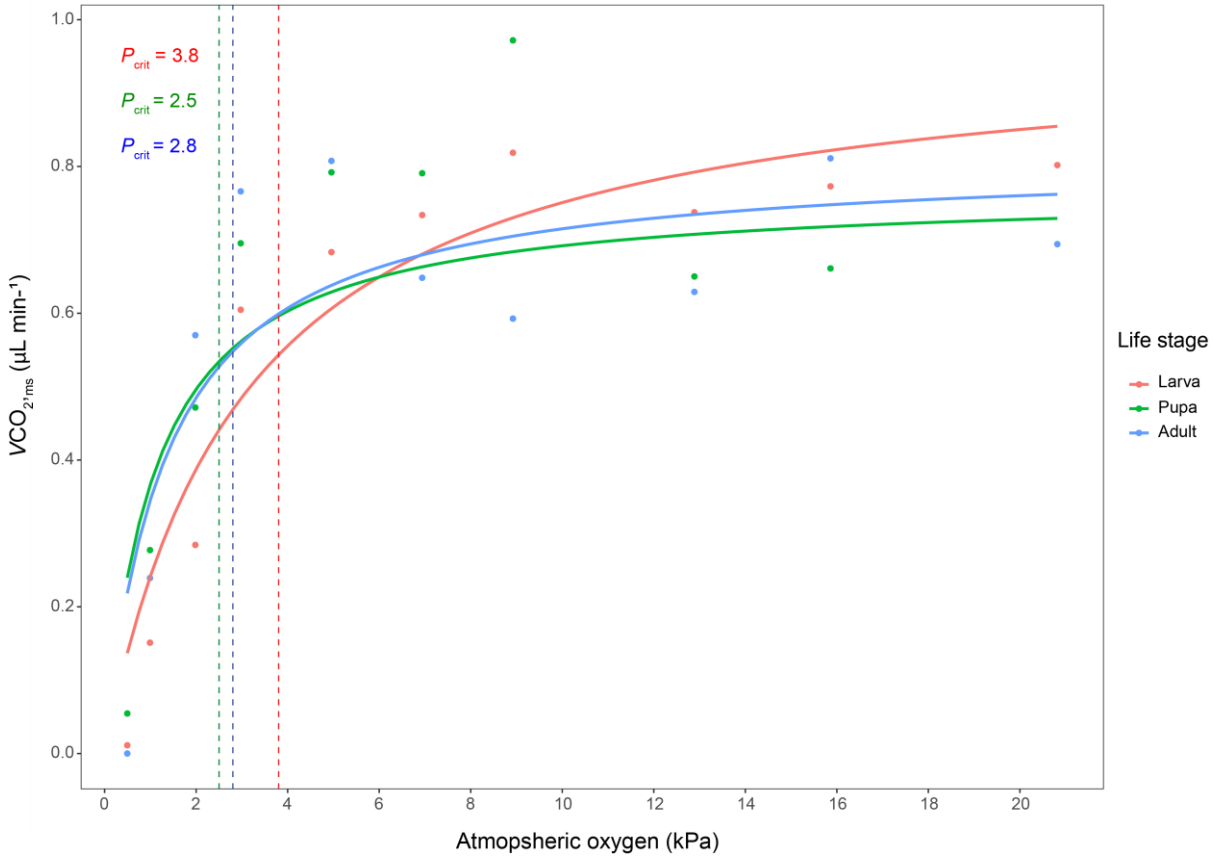

Fig. 5 Mass-corrected volume of carbon dioxide output ($\dot{V}CO_{2,ms}$) by *Hylurgus ligniperda* (F.) in response to a stepwise reduction in partial pressures of oxygen (P_{O_2}) (21–0.5 kPa) at 20°C modelled with nonlinear least squares regression. Dashed vertical lines indicate the mean critical P_{O_2} (P_{crit}) threshold for each life stage below which metabolism abruptly drops.

Pupae had the highest capacity to utilise available O_2, with an average (± SEM) K_m of 1.23 ± 0.31 kPa, followed by adults 1.50 ± 0.21 kPa, and then larvae 3.70 ± 0.92 kPa (Fig. 6C). This is congruent with the $\dot{V}CO_{2,max}$ (maximum $\dot{V}CO_2$ measured at normoxia), with larvae highest at 1.03 ± 0.11 μL h^{-1} mg^{-1}, followed by adults 0.82 ± 0.03 μL h^{-1} mg^{-1} and then pupae which had the lowest $\dot{V}CO_{2,max}$ of 0.79 ± 0.08 μL h^{-1} mg^{-1} (Fig. 6B). Adult *H. ligniperda* theoretically had the most efficient tracheal conductance for O_2 with a G_{max} (in μL h^{-1} mg^{-1} kPa^{-1}) of 0.19 ± 0.01, followed by larvae 0.14 ± 0.02, and then pupae 0.10 ± 0.01 (Fig 6D).

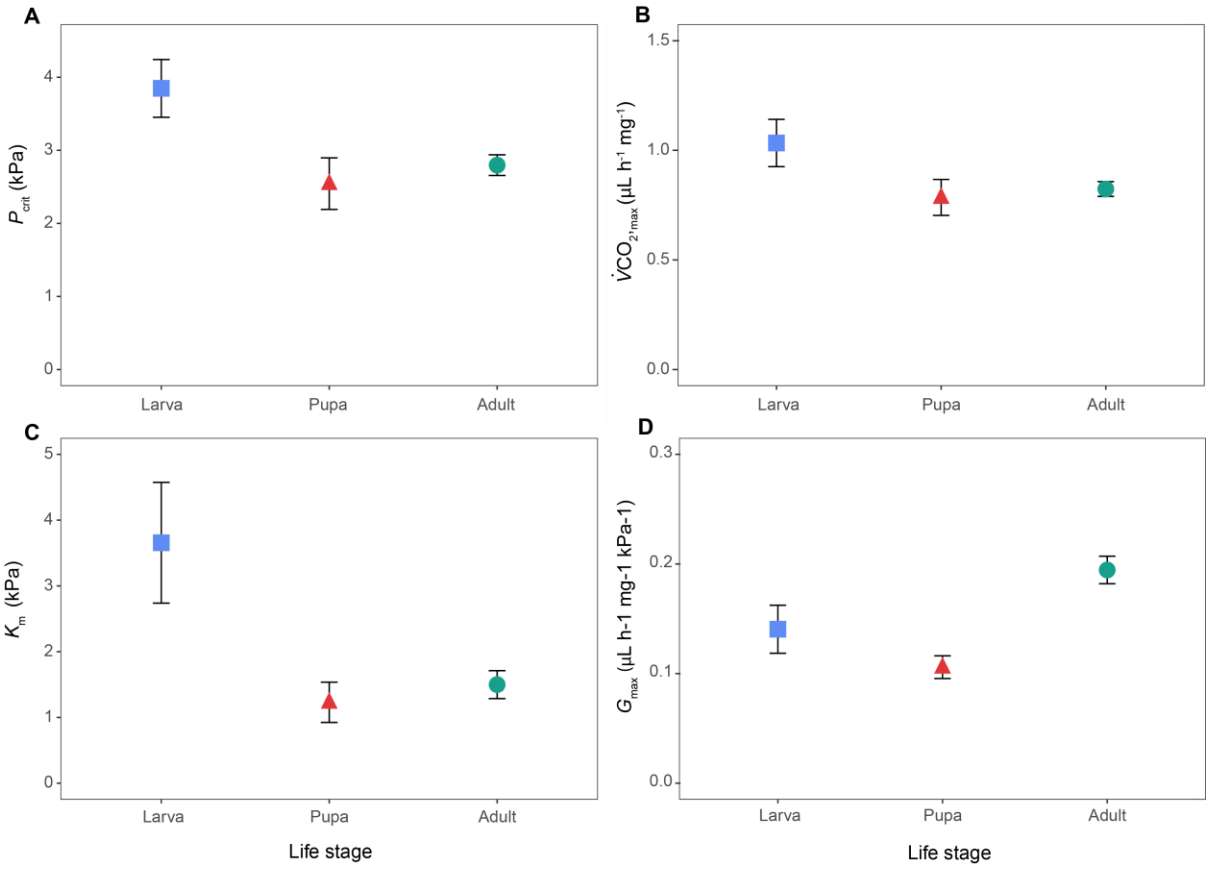

Fig. 6 Average ± SEM respiratory responses of each life stage of *Hylurgus ligniperda* (F.) at 20°C. **A)** is the partial pressure of oxygen critical threshold (P_{crit}), **B)** is the maximum CO_2 emission at normoxia ($\dot{V}CO_{2,max}$), **C)** is the partial pressure of oxygen at which $\dot{V}CO_2$ is 50% of maximum (K_m), and **D)** is the indicated tracheal system's maximal conductance for O_2 (G_{max}).

3.4 Discussion

We found that *H. ligniperda* respiration was consistent with a continuous ventilatory pattern at each life stage; there was no clear evidence of a discontinuous gas exchange or other cyclic respiratory pattern. However, the continuous pattern observed could be an artefact of the small size of the individual insects and the minute $\dot{V}CO_2$ output, making it difficult to distinguish other patterns (Terblanche & Woods, 2018). This study supports our hypothesis that $\dot{V}CO_2$ decreased at the lowest temperature of 10°C and increased at 20°C for all life stages. Further, our prediction that pupae would be the least affected by hypoxia was supported. At 10°C, there was negligible variation in $\dot{V}CO_2$ emission from pupae between the O_2 treatments. We also found that pupae had the lowest P_{crit} in response to a stepwise reduction in O_2, indicating that they can potentially regulate their internal P_{O_2} under severe hypoxia (~ 2.5 kPa). The $\dot{V}CO_2$ of *H. ligniperda* adults and larvae increased with hyperoxia and decreased with hypoxia at 20°C, whereas $\dot{V}CO_2$ of pupae decreased at 20°C under hyperoxia. All life stages showed negligible responses within their own group to changes in atmospheric conditions at 10°C.

The calculated P_{crit} values indicate that all *H. ligniperda* life stages have the ability to regulate their need for O_2 (oxyregulation) under extremely low ambient O_2 levels, up until a certain point. The point at which each life stage switched to conform to the ambient P_{O_2} levels (oxyconformation), was lowest for pupae (2.5 kPa), followed by adults (2.8 kPa), and then larvae (3.8 kPa). This indicates that pupae may have the highest safety margin for hypoxia, meaning they can regulate their internal P_{O_2} at extremely hypoxic levels (~ 2% O_2), whereas larvae had the smallest safety margin (~ 4% O_2). Pupae also had a significantly higher binding affinity for O_2 (1.23 kPa) compared to larvae, which had the lowest affinity for O_2 (3.70 kPa). A high-affinity ligand binding for O_2 results from greater attractive forces between O_2 and its receptor, indicated here by a low $K_{m.}$ Although pupae were the most efficient oxyregulators, they had the lowest calculated maximal tracheal conductance (G_{max}) out of the life stages. This could be due to pupae being in a dormant immobile state, leading to a decreased need to have a high conductance for O_2 (Greenlee & Harrison, 2004). Adults had a significantly more efficient tracheal conductance when compared to pupae. Increased tracheal conductance with an increase in age/developmental stage was observed in the American locust (*Schistocerca americana*), likely because of development toward flight in the adult (Greenlee & Harrison, 2004). Similarly, *H. ligniperda* adults are highly mobile, with the ability to fly several kilometres (Meurisse & Pawson, 2017), which would likely require higher O_2 delivery than

less mobile life stages (Socha et al., 2010). Pupae, on the other hand, may rely more on passive gas diffusion compared to larvae and adults, as is seen in other small developing insects. Passive gas diffusion reduces the need for a more robust tracheal system (Pendar et al., 2015). Pupae do display abdominal pumping (pers. obs.) however, which may be a sign of active ventilation (Pendar et al., 2015).

At 10°C the CO_2 release of all life stages of *H. ligniperda* was less sensitive to atmospheric changes. The lack of any significant effect from the different atmospheric treatments at 10°C is likely a result of reduced metabolism at this temperature (Lee, 1991). Mitochondrial function decreases at lower temperatures, decreasing ATP production, hence slowing metabolism (Angilletta, 2009). Exposure to low temperatures can have a protective effect on insects (Boardman et al., 2013). Under low temperatures the switch to anaerobic metabolism when exposed to hypoxia is less detrimental (Boardman et al., 2016). For instance, with *Drosophila melanogaster*, rearing the insects long-term under hypoxia (10% O_2) at 15°C, had little effect on development compared to temperatures > 16°C (Frazier et al., 2001).

The response from *H. ligniperda* at 20°C to the changing atmospheric conditions was more apparent. $\dot{V}CO_2$ output increased under a hyperoxic atmosphere (40 kPa) in larvae and adults, indicating an increase in metabolism, whereas $\dot{V}CO_2$ dropped in *H. ligniperda* pupae. Similarly, in the dragonfly *Erythemis simplicicollis* hyperoxia (30 and 50% O_2) significantly increased metabolism (Harrison & Lighton, 1998), increased $\dot{V}CO_2$ output of *Bombyx mori* larva (Boardman & Terblanche, 2015), and increased $\dot{V}CO_2$ output in *Manduca sexta* eggs (Woods & Hill, 2004). The reduction in pupal $\dot{V}CO_2$ output in response to hyperoxia could be a result of a decrease in active ventilation to guard against the deleterious impacts of reactive oxygen species from a higher O_2 atmosphere (Harrison et al., 2006). Under hypoxia *H. ligniperda* adults $\dot{V}CO_2$ decreased by ~ 50% from normoxia. Hypoxic atmospheres directly limit ATP production, depressing metabolism (Harrison & Haddad, 2011; Cao et al., 2019). Hypoxia impacts on insects are more evident at warmer temperatures (Boardman et al., 2015). The synergistic effects of higher temperatures and hypoxic atmospheres are well highlighted in modified atmosphere pest control experiments (Donahaye et al., 1996; Navarro, 2012; Neven et al., 2014). For example, at 26°C in 1% O_2, it took ~ 6 days to kill 99% of *Tribolium castaneum* adults compared to almost 2 days to achieve the same mortality at 36°C (Donahaye et al., 1996). *H. ligniperda* larva also decreased $\dot{V}CO_2$ output in response to hypoxia at 20°C (~ 20%), whereas pupa showed a negligible response (~ 5% decrease).

The responses of *H. ligniperda* to the O_2 and temperature treatments, along with their relatively low P_{crit} thresholds, could have important implications for bark beetle management. At both temperatures pupae had lower $\dot{V}CO_2$ output than larvae and adults, indicating a lower metabolic rate. We suggest pupae probably have a higher tolerance to some control methods compared to the larval and adult life stages of *H. ligniperda*. For instance, fumigants that require active respiratory uptake (e.g. phosphine; Nayak et al., 2020), or reduce O_2 (e.g controlled atmosphere disinfestation; Ahn et al., 2013), or metabolic suppression (e.g. cold treatments; Boardman et al., 2015), would likely be less effective against pupae because of their low metabolic rate. Further, insects with the ability to withstand low O_2 atmospheres are more tolerant of phosphine (PH_3) (Cheng et al., 2003; Nath et al., 2011), partly because a switch to anaerobic respiration limits PH_3 uptake (Schlipalius et al., 2006). Similarly, tolerance to controlled atmosphere disinfestation has been noted in insects already adapted to low O_2 environments (Hoback, 2012). The bark beetle *Hypothenemus obscurus*, a pest of macadamia nuts, is also resistant to low O_2 levels, and as a result, can withstand anoxic conditions (0% O_2) for up to 14 days before 100% mortality is achieved (Delate et al., 1994).

Larva and adult *H. ligniperda* showed marked metabolic responses to both increased O_2 and increased temperature. We suggest that increasing their metabolic rates via the application of super-atmospheric O_2 levels (i.e. above ambient > 20.9% O_2) and/or increasing temperature could complement the efficacy of some control methods. An increase in temperature had a significant positive effect on PH_3 efficacy in the control of *Liposcelis bostrychophila* (Nayak & Collins, 2008). Similarly, the addition of hyperoxic levels of O_2 (> 40%) directly correlated with an increase in mortality from PH_3 against four different species of insects (Liu, 2011). Increased temperature and/or increased O_2 have also been shown to complement other control techniques, such as increasing insect mortality in controlled atmosphere disinfestation via increased temperature (Whiting & Hoy, 1997), and phytosanitary irradiation can be enhanced through increasing O_2 levels above ambient (Hallman et al., 2016).

3.5 Conclusion

The results from this study highlight the importance of understanding fundamental aspects of insect physiology. We found strong support for our hypothesis that *H. ligniperda* regulate their O_2 levels under severe hypoxia, likely as a consequence of their endophagous life history. Further, our results supported our prediction that pupae would display the lowest metabolism compared to the two other life stages. Both larvae and adults had comparatively higher metabolic rates, which correlates with these two life stages being more mobile than pupae. Larvae and adults were also more sensitive to atmospheric changes, eliciting dynamic responses to both hypoxic and hyperoxic conditions at 20°C. We posit that control treatments could be less effective on bark beetle pests, particularly at the pupal stage, as a result of their ability to regulate their need for O_2 under severely hypoxic atmospheres.

Chapter 4 - Phosphine toxicity varies with life stage in the forest insect, *Hylurgus ligniperda* (F.)

4.1 Introduction

The importation of wood and plant products is a major vector pathway for forest pests (Hulme, 2009; Roy et al., 2014; Hurley et al., 2016) resulting in the establishment of thousands of invasive species worldwide (Meurisse et al., 2019). Over the last several decades there has been extensive use of methyl bromide (MB), a broad-spectrum fumigant effective against many unwanted organisms (i.e. insects, fungi, bacteria) (Methyl Bromide Technical Options Committee, 2018). MB has been crucial in the quarantine and pre-shipment (QPS) space as it is an accepted disinfestation tool (Taylor, 1994). However, MB is a noted ozone depletory and as a result there is global agreement to reduce the release of MB into the atmosphere (Methyl Bromide Technical Options Committee, 2014).

The trade in unprocessed whole logs is particularly challenging for treating unwanted organisms because of the large volumes of material transported. Further, the lack of processing negates any mechanisms which could otherwise kill many pests, such as debarking and drying (Liebhold et al., 1995). New Zealand's largest use of MB currently is for the QPS treatment of export wood products, predominantly unprocessed whole radiata pine (*Pinus radiata* D.Don) logs for export (Ministry for Primary Industries, 2019a). China is the largest importer of pine logs from New Zealand and, to reduce the use of MB, an experimental agreement in 2001 was reached between New Zealand and China. This allowed for pine logs transported below deck to be treated with phosphine (PH_3) instead of MB (Genera Biosecurity Ltd, 2020; United Nations Environment Programme, 2016b). Approximately 70% of logs exported to China are currently treated with PH_3 (Ministry for Primary Industries, 2019a). Pine logs are fumigated with PH_3 for 240 hours in-hold (i.e. an enclosed space below deck) during transit from New Zealand to China. The fumigation schedule is an initial dose of 2 g/m^3, followed by an additional 1.5 g/m^3 top-up at 120 hours; this schedule was based primarily on an efficacious PH_3 schedule for grain pests in Australia (Frontline Biosecurity Ltd, 2004; Brash & Page, 2009). 200 ppm (~ 0.1 g/m^3) is the stipulated minimum concentration of PH_3 required within each hold per day during the fumigation period (Ministry for Primary Industries, 2018b). On-board monitoring of PH_3 has shown that the holds for the most part maintain a concentration

of ≥ 200 ppm during the fumigation period as recommended (Hall et al., 2016). After 240 hours the ship holds are ventilated whilst still at sea.

Phosphine is a slow-acting insecticide that requires active respiratory uptake by insect pests, whereas MB penetrates the insect's cuticle (Price, 1985). This difference in the mode of action between PH_3 and MB may be a potential cause in the differences seen in efficacy between the two fumigants. MB is noted as being generally more effective than PH_3 against insect pests (Heather & Hallman, 2008; Williams et al., 2000). PH_3 has been in use for the disinfestation of stored food products for several decades, and it is considered relatively effective in this area (Daglish et al., 2018). However the misuse of PH_3, predominantly through use in non-gastight areas or using sublethal concentrations, has led to insecticide tolerance developing in some insect populations (Holloway et al., 2016). Nevertheless, PH_3 is considered an effective, low-residue fumigant, and importantly, it is not an ozone-depleting substance (Ryan & Lima, 2014).

Bark beetles (Coleoptera: Scolytidae) are a common type of forest pest that typically burrow into the cambium of host trees, where they live out the majority of their life cycle (Raffa et al., 2015). They are easily introduced to novel areas through the transport of wood products (Allen et al., 2017). Three common forest pest insects of radiata pine found in New Zealand are *Hylastes ater*, *Arhopalus ferus*, and *Hylurgus ligniperda*; these species, amongst others, are listed as unwanted organisms in the import standards for China (Ministry for Primary Industries, 2000). There is little published literature on the efficacy of PH_3 on bark beetle or wood boring insects, or on how PH_3 behaves when used in pine log disinfestation. PH_3 did control the Japanese termite (*Reticulitermes speratus*) during a 7-day fumigation at 5°C (Choi et al., 2014) and was also effective in controlling partially exposed (i.e. insects within bark chips) *H. ater* adults and larvae, and *A. ferus* adults and eggs, at 200 ppm over a 10 day period at 15-18°C (Zhang et al., 2004). Nonetheless, there were unconfirmed reports that live bark beetles, particularly *H. ligniperda*, were intercepted at Chinese ports post PH_3 fumigation, suggesting that the New Zealand PH_3 schedule may not be 100% efficacious (Armstrong, Brash, Waddell, et al., 2014; Zhang et al., 2004). PH_3 efficacy for disinfestation has shown to be highly variable depending on the target insect species, temperature, the duration of the fumigation, the concentration, and the atmospheric conditions in which the fumigation is taking place (Armstrong, Brash, Waddell, et al., 2014; Chaudhry, 1997).

It is more difficult to achieve 100% mortality using PH_3 in low temperatures (≤ 10°C) compared to higher temperatures (≥ 15°C) (Bond & Monro, 1984; Kaur & Nayak, 2015). For instance,

PH_3 fumigations at higher temperatures increased mortality in the booklouse *Liposcelis bostrychophila*, irrespective of PH_3 concentration (Nayak & Collins, 2008). Decreased insect metabolism at lower temperatures causes a decrease in aerobic respiration. A reduction in the uptake of PH_3 at low temperatures due to a decrease in insect respiration has been posited as a likely mechanism behind the reduced mortality (Chaudhry et al., 2004; Hetz, 2010).

Moisture content of the commodity being treated is another factor that can have an impact on PH_3 effectiveness (Reddy et al., 2007). A positive correlation between PH_3 sorption and increasing moisture has been shown (Reddy et al., 2007; Daglish, & Pavic, 2008). This means that grain or any commodity with a higher moisture content tends to have more absorptive abilities, leading to a decrease of PH_3 within the headspace atmosphere, which potentially leads to a decrease in efficacy (Hall et al., 2018; Pranamornkith et al., 2014; Reddy et al., 2007). Recently harvested wheat with a moisture content of 8.5% absorbed 1.6 times more PH_3 than older, commercially available wheat (Daglish & Pavic, 2008). The average moisture content of freshly felled radiata pine logs with bark cover is over 100%, based on a dry basis moisture content measurement (Devitt et al., 2020; Hall et al., 2018), and this is likely comparable to the radiata pine logs exported from New Zealand to China.

The duration of exposure time to PH_3 is also an important factor in its efficacy, perhaps more so than any other major fumigant. PH_3 does not tend to follow a typical concentration x time relationship. Typically, a higher dose of a toxin over a short period is equivalent to a lower dose over a longer period (Guedes et al., 2016; Guedes et al., 2017). However, moderate to low doses of PH_3 over longer periods are more effective than high doses over short periods (Bond & Monro, 1984; Chaudhry, 1997). Some studies indicate that high doses of PH_3 over short periods can lead to a 'narcotic' response in some insect species (Woodman et al., 2008; Chaudhry, 1997; Winks, 1984). Narcosis, or the shutdown of metabolic processes, may afford protection from PH_3 because respiratory uptake is significantly reduced in this state (Cao & Wang, 2001; Malekpour et al., 2019). Similarly, low oxygen (O_2) environments can afford some measure of protection from PH_3 for certain insect pests as low O_2 also reduces respiration (Liu, 2011). Conversely, the addition of O_2 during the initial application of PH_3 can dramatically increase PH_3 efficacy (Liu, 2011; Liu et al., 2015).

Phosphine has been effective over shorter durations in exposed (uncovered) insects. A 100% mortality was achieved for exposed *H. ligniperda* and *H. ater* larvae, with 72-hour fumigations at 15°C, with a dose of 700 ppm; however, sample sizes were small (n = 5-8) (Brash et al.,

2008). Similarly, later bioassays indicated good control with mean PH_3 doses ranging from 260 to 3,180 ppm over 3-7 days in duration for *H. ligniperda* and *H. ater*, although again sample sizes were small ($n \leq 10$), and the ability to detect differences was low (Brash & Page, 2009). One in-log study (i.e. insects within the cambium of pine logs) at 24-26°C following the commercial schedule (2 g/m^3 + 1.5 g/m^3 top-up) achieved 100% mortality for *H. ater*, *H. ligniperda* and *A. ferus* adults, larvae and pupae (Frontline Biosecurity Ltd, 2004). However, subsequent studies reported mixed results with higher doses (3-4 g/m^3), with *H. ligniperda* potentially surviving (insects were noted as 'moribund' i.e. about to die, rather than dead) after 72-120 hour fumigations at 15°C (Brash & Page, 2009).

In this study, I evaluated the efficacy of PH_3 against bark beetles in laboratory settings. I selected the invasive bark beetle *H. ligniperda* as my test species as it is frequently intercepted worldwide on wood products (Haack, 2006; Zhang et al., 2011; Brockerhoff, Bain, et al., 2006). This species is commonly found in New Zealand (Brockerhoff et al., 2006), has a high risk of establishment in China (Yu et al., 2019), and has been intercepted in China from New Zealand exports (Zhang et al., 2011). Moreover, this species is readily available in the field and has been successfully reared in the laboratory (Clare & George, 2016).

I aimed to establish a clear dose-response relationship between the toxicity of PH_3 to *H. ligniperda* at both 10°C and 20°C, across all life stages. I selected 10°C, as this temperature is the average lower temperature experienced at the Port of Tauranga (National Institute of Water and Atmospheric Research, 2016) where 40% of log exports depart (TENCO Ltd, 2020). Further, temperatures below 15°C have been recorded during in-transit PH_3 fumigations (Hall et al., 2016). I selected 20°C to represent average ambient temperatures at the Port of Tauranga (National Institute of Water and Atmospheric Research, 2016).

Firstly, I administered a series of 72-hour rangefinder fumigations to establish which PH_3 concentrations to test against exposed (removed from cambium) life stages of *H. ligniperda*. Rangefinder fumigations consist of a series of doses, from low to high, in order to establish a dose-response curve (Ritz & Streibig, 2005). I then undertook longer duration rangefinder experiments (120-240 hours) as initial 72-hour experiments did not achieve a clear dose-response relationship.

I did a series of rangefinder experiments on insects that were still within the cambium (in-log) to assess PH_3 efficacy in a more realistic scenario, however again a clear dose-response relationship was not established. Finally, I tested the New Zealand commercial fumigation

schedule for PH_3 treatment of pine logs under what were deemed favourable conditions (hereafter called the commercial scenario) based on my earlier experiments.

4.2 Methods

4.2.1 Insect colony

I established a new *H. ligniperda* colony from both laboratory-grown (provided by Plant and Food Research (PFR) colony, Mt Albert, Auckland) and field-collected insects from the Manawatū area, North Island, New Zealand. The PFR Mt Albert colony was established in 2012 from insects collected from the Central North Island and Canterbury regions, New Zealand (Clare & George, 2016). Care was taken to ensure insects were evenly mixed from both laboratory-grown and field-caught insects when establishing the new colony. The new colony was kept at 20°C in darkness and maintained *ad libitum* on artificial diet (See 'diet 5' in Barrington et al., 2015) provided by PFR.

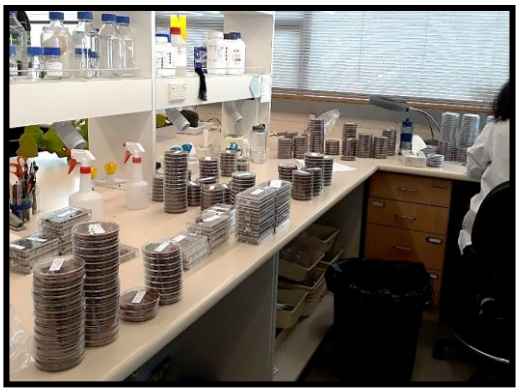

Fig. 1 Top: The *Hylurgus ligniperda* (F.) (n ~ 1000) colony housed within petri dishes and tissue culture trays. **Bottom left:** hand-made corrugated plastic 'sandwiches' being prepared for adult *H. ligniperda* to lay eggs in for collection. **Bottom right:** artificial insect diet after being treated with ultraviolet light to kill microbes.

4.2.2 Exposed insect rangefinder fumigations

Egg preparation

Eggs were hand-collected daily from custom corrugated plastic 'sandwiches' using a small damp brush (Fig. 1). Eggs were then placed on moistened paper within petri dishes and stored at 10 ± 1°C to halt development until they were needed for fumigation (Clare & George, 2016).

To prepare the eggs for fumigation, 5 rows of 10 eggs (n = 50) were placed within petri dishes (90 mm x 15 mm) containing Phytagel™ (Sigma-Aldrich®, Auckland, New Zealand), approximately 2-3 mm in depth. Before fumigation eggs were acclimatised to their respective experimental temperatures, either 10 or 20°C, for 24 hours. When ready for fumigation the lid of the petri dish was removed to allow for PH_3 exposure, then replaced after degassing.

Larvae, pupae, and adult preparation

Third and fourth instar larvae were selected for fumigation (n = 25-50) and placed in individual 3 ml 24-well tissue culture plates (Fig. 2) (CC tray) (Interlab®) containing approximately 3 g of crumbled insect diet per well. Similarly, first and second instar pupae (n = 25-50), and fully mature (e.g. hard exoskeleton and dark brown) adult *H. ligniperda* (n = 25-50) were selected, prepared and acclimatised in the same manner as the larvae. Custom wire mesh lids replaced the plastic CC tray lids during fumigation to contain insects and allow for PH_3 penetration.

Fig. 2 *Hylurgus ligniperda* (F.) pupae in tissue culture trays after fumigation with phosphine (PH_3).

4.2.3 Fumigation

Insects (n = 25-50) were placed in 28 L airtight fibreglass chambers (Labconco® desiccators, Kansas City, Missouri, USA) and then placed within a temperature-controlled shipping container (Transicold Thinline). The chambers were maintained at either 10 ± 1 or 20 ± 1°C during fumigation. A small, dampened sponge (75 mm x 75 mm x 10 mm) was placed within each 28 L chamber to help combat insect desiccation during fumigation. Chambers were then fumigated with PH_3 (1.4% PH_3, in nitrogen balance, BOC, Australia; except for the control) with their respective doses using a 1-litre gas-tight syringe (Hamilton Co., Reno, Nevada). After 72- or 240-hours, chambers were degassed for approximately 2 hours, and insects were then placed under a fume hood for 12 hours before further processing.

4.2.4 Gas chromatography

A SRI 8610C (Mandal, USA) gas chromatograph (GC) was used to measure the concentration of PH_3 in the headspace of each chamber. Headspace samples of 3 ml were drawn from the chambers using 5 ml gas-tight syringe (Valco Instruments Co., Texas, USA), and each sample was immediately injected into the GC column with a sample run time of 3 minutes. Samples were drawn once daily at approximately the same time of day (± 60 minutes) over the fumigation period of 72 or 240 hours.

The GC was fitted with a flame photometric detector (FPD) set to 300°C with a CTR-1 packed column (1,828.8 mm x 6.35 mm, 9' MS5A and 6' Hayesep D). Four to five-point calibration curves for PH_3 (1.4% PH_3, in nitrogen balance, BOC, Australia) were established at the start of each experiment using a range of dilutions. The configuration of the GC included: helium (BOC, New Zealand, instrument grade > 99.99%) 60 psi, hydrogen (BOC, New Zealand, instrument grade > 99.98%) 30 psi, air (BOC, New Zealand, dry, O_2 21.0 ± 1.0% in nitrogen balance) 3 psi.

4.2.5 Mortality bioassay

Eggs

After fumigation, petri dishes containing eggs were stored at 20°C in darkness. Mortality was assessed under a compound microscope with hatched eggs counted as alive, and unhatched counted as dead. The first assessment was at 24 hours post fumigation, then subsequently every 48 hours until a final assessment at 240 hours.

Larvae, pupae, and adults

All insects were stored as above for eggs. Larvae, pupae, and adults were removed from their original diet and placed on fresh artificial diet within tissue culture trays to remove exposure to any PH_3 residue. Mortality was first assessed at 24 hours post fumigation for all life stages, then every 24 hours for larvae and adults up to 96 hours. For pupae, mortality counts were undertaken after the first 24 hours, then every 48 hours, with the final assessment done at 240 hours. Insects were gently prodded with a small brush to check for movement, any movement was counted as alive, prolonged lack of movement was counted as dead. Progression from one life stage to the next i.e. pupae emergence to adult was also counted as alive.

4.2.6 In-log rangefinder fumigations

Insects

Laboratory grown insects were received as mating pairs from PFR Mt Albert on a fortnightly basis to infest cut radiata pine logs.

Log preparation and infestation

Freshly cut radiata pine logs (240 mm diameter x 300 mm height approx.) were end-grain sealed using paraffin wax (Fig. 4) (see Pranamornkith et al., 2014) and stored at 10°C. Logs were prepared for infestation with *H. ligniperda* by placing damp paper towels on the bottom of an open plastic bag. The log was then placed on top of the damp paper towels, and then a rolled 'skirt' of dampened paper towels was wrapped around the base of the log (Fig. 3). Fifteen adult mating pairs (female n = 15, male n = 15) of *H. ligniperda* were placed on the top of the log and the bag sealed with a twist tie (Fig. 4); this process was repeated until ~ 50 of logs were infested at a time. The infested logs were stored at 20 ± 1°C for several weeks until a selected life stage was needed. Bags were aerated once after the first week and then fortnightly by opening the top of the bag, flushing the air and re-sealing.

Fig. 3 Diagram showing setup of radiata pine (*Pinus radiata* D.Don) log infestation with *Hylurgus ligniperda* (F.). Illustration, Devitt, 2020.

Fig. 4 Left to right: paraffin wax-sealed logs before the infestation process. A *Hylurgus ligniperda* (F.) infested log within a black plastic bag. Planed infested log ready for fumigation in the 28 L chamber (Labconco® desiccators). *Pinus radiata* (D.Don) log post-fumigation with bark and insects removed for mortality assays.

Log preparation: pre fumigation

Infested pine logs for fumigation were selected based on the life stage needed for each experiment (i.e. larvae ~ 6-7 weeks, pupae ~ 8 weeks, adults ~ 10-12 weeks, post infestation date). Selected logs were acclimatised to the experimental temperature (10°C) for 24 hours before the fumigation. Acclimatised logs were removed from the plastic bags, and the waxed ends planed (Bosch, DIY Planer 83 Mm 550 watt) to expose the wood underneath. Logs were then placed within 28 L fibreglass chambers (Labconco® desiccators, Kansas City, Missouri, USA) and fumigated as per exposed insect fumigations for a duration of 240 hours. The logs were then degassed for 12 hours after the 240-hour fumigation. PH_3 sampling and GC measurements were conducted as per exposed insect fumigations.

Log preparation: post fumigation

Once logs had been degassed, they were removed from the chambers and the bark was carefully removed by hand (Fig. 4). Insects within the bark were carefully removed by breaking the bark into smaller pieces, as well as removing the insects from the surrounding frass present on the log with a small brush.

4.2.7 Mortality bioassays – all experiments

All life stages were first counted 24 hours post fumigation, and then every 48 hours, up 240 hours, with 240 hours being the final mortality count.

Larvae and adults

Once removed from the bark, live larvae were placed on an approximately 3 mm thick layer of lightly packed crumbled diet in petri dishes. Dead larvae were placed in an empty petri dish, however, if they were found to still be alive during a later count they were then moved to a petri dish with artificial diet. Progression from larvae to pupae was counted as alive.

Pupae

Live pupae were placed within tissue culture trays with approximately 3 g of compacted crumbled diet within each well. Dead pupae were placed in petri dishes with a 3 mm layer of insect diet crumbled then lightly compacted within the dish. If 'dead' pupae displayed movement at any point they were moved to the tissue culture tray, if pupae displayed no movement via prodding with a soft brush each day they were removed from the live tray and placed in the petri dish. Progression from pupae to adults was counted as alive.

4.2.8 Commercial scenario experiment

Log infestation, and preparation pre and post fumigation

Log infestation, as well as the preparation prior to and the removal of bark after fumigation, was done using the same methodology as described above for the in-log rangefinder fumigation experiments.

Experimental setup

A randomised block design was implemented for the commercial scenario experiment. Sixteen *H. ligniperda* adult infested pine logs were randomly assigned to either the no top-up group (n = 8 logs) or the top-up group (n = 8 logs) (Fig. 5). Within each group, the eight infested logs were then assigned as either a control or a treatment log. Hence for each group, there were four logs treated with PH_3 and four logs not exposed to PH_3 (Fig. 5). Control logs were placed within the 28 L airtight chambers (Fig. 4) (Labconco® desiccators, Kansas City, Missouri, USA) at the same time as the treated logs and remained sealed for the same amount of time as their respective treated logs within their group. Control logs were also degassed when treated logs within the group were degassed. This experiment was repeated 3 times.

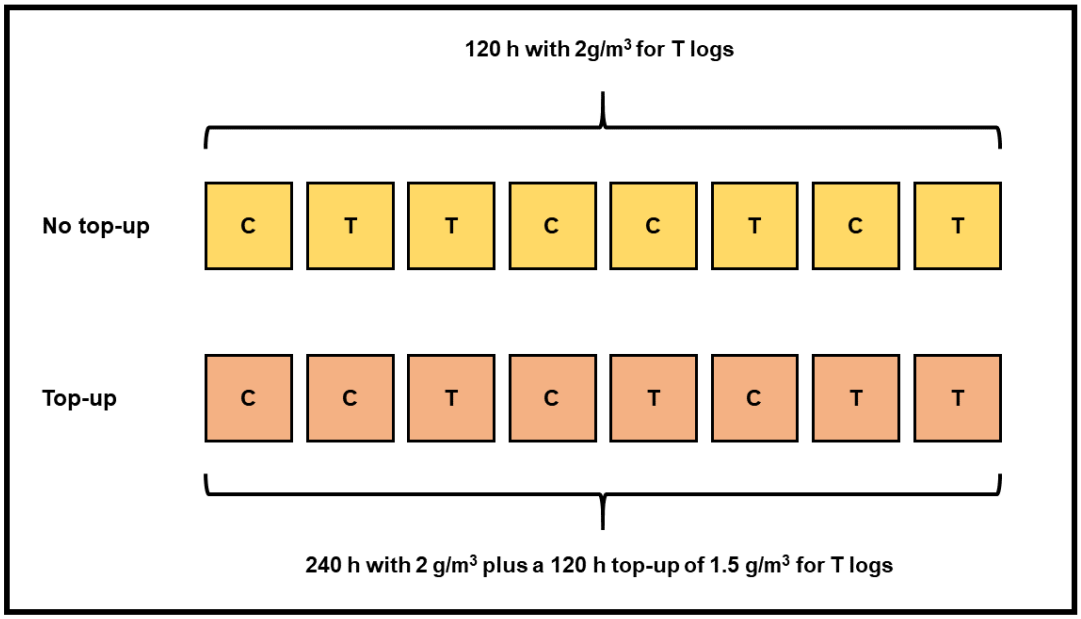

Fig. 5 Diagram depicting the commercial scenario experimental setup. **T** indicates logs treated with phosphine (PH_3) and **C** indicates control logs. **No top-up group:** 120-hour fumigation with PH_3 at a dose of 2 g/m^3 for the entire 120-hour duration. **Top-up group:** 240-hour fumigation with PH_3 with an initial dose of 2 g/m^3 followed by a top-up dose of 1.5 g/m^3 of PH_3 at 120 hours.

Table 1. List of experiments undertaken in assessing the toxicity of phosphine (PH_3) to all life stages of *Hylurgus ligniperda* (F.) under different temperatures, durations, and dose ranges.

Experiment		No. replicates	Life stage	Temp (°C)	Duration (h)	Dose-range (ppm)
Exposed insects		1	Eggs, larvae	10	72	0-400
			Pupae, adults	20	72	0-350
		1	Pupae	10	120	0-1400
		1	Larvae, adults	10	240	0-160
		3	Pupae	10	240	0-1400
In-log	**Narrow dose range**	1	Larvae, pupae Adults	10	240	0-300
	Extended dose range	1	Larvae, pupae Adults	10	240	0-1400
Commercial scenario	**No top-up group**	3	Adults	20	120	0-1400
	Top-up group	3	Adults	20	240	0-1400

4.2.9 Statistical analysis

All exposed insect rangefinder experiments had a fixed number of individuals per dose – either 25 or 50 depending on the experiment and life stage. For the in-log and commercial scenario experiments, there were varying and uncontrollable sample sizes per dose due to the varying levels of insect infestation in each log and/or the timing accuracy for life stage selection. There were several instances where sample sizes were very low in-log (i.e. $n < 20$; Ritz & Martinussen, 2011). Therefore, replicates with < 10 individuals were removed from the datasets before analysis based on best practice in the literature (Peduzzi et al., 1996).

Data from the different experiments was tested for normality (Gaussian distribution) through visual inspection of quartile to quartile plots as well as Shapiro-Wilks test for normality. The data varied in normality depending on the experiment, with all experiments having a normal distribution, with the exception of the commercial scenario experiment.

For statistical analysis, the treatment dose was entered as ppm, and the mortality of insects was entered as the proportion of dead insects divided by the total insects within each sample. Proportional mortality data were analysed using generalised linear models (GLM) with a binomial family distribution and a probit family link using R statistical software (v.3.6.1 R Core Team 2019). A logit link was explored for the commercial scenario experiment due to the non-normal distribution of the data (Finney, 1952). However, both the Akiake information criterion (AIC) and pseudo-R^2 showed a better fit for the data modelled with a probit link versus a logit link, thus the probit link was used. The most parsimonious GLM for each dataset was selected by visual assessment of quartile to quartile plot residuals, and residual deviance versus predicted deviance using the *DHARMa* package (v.0.2.4 Hartig, 2019), as well as selecting the model with the lowest scoring AIC (Burnham & Anderson, 2002). Further, the Aldrich-Nelson pseudo-R^2 (Aldrich et al., 1984) with the Veall-Zimmerman correction (Veall & Zimmermann, 1994) was used to assess the explanatory power of each GLM used in its respective dataset, which is suggested as the most reliable pseudo-R^2 for binary data (Veall & Zimmermann, 1994; Signorell, 2020) from the *DescTools* package (v.0.99.30 Signorell et al., 2019). Spearman correlation coefficient (r_s) was used to assess the relationship between dose, duration, temperature, and mortality.

Concentration over time was calculated as follows:

$$\text{CT Product} = \left[\frac{\text{Application rate + Minimum concentration}}{\text{Number of concentration readings}} \right] \text{x Exposure Period}$$

Where percentage mortality is reported in the text, the standard error of the proportion (SE_p) was calculated SE_p = sqrt[p(1-p)/n], where p is the proportion and n is the number of observations in the sample. Where appropriate, post-hoc pairwise analysis of variables from significant GLM models was done using the *emmeans* package (v.1.4.1 Lenth et al. 2018), which uses estimated marginal means to assess differences between the independent variables - life stage, replicate, temperature, and fumigation length against the dependent response

variable (proportion dead). All graphs were created using the *ggplot2* package (v. 3.2.1 Wickham & Winston, 2019).

I calculated the probability of a lethal dose (LD) to achieve 50% (LD_{50}) and 99% (LD_{99}) mortality from the GLM model along with asymptotic standard error for each dataset using the *MASS* package (v.7.3-51.4 Ripley et al., 2013). Since a typical sigmoidal dose-response relationship was not achieved for the majority of the bioassays there was a high margin of error in the projected LDs for most datasets. This coupled with small samples sizes by toxicity modelling standards ($n < 100$) (Rainey & McKaskey, 2015) meant that the confidence intervals for the calculated theoretical LD_{99}'s were high, which is typical in smaller datasets (Rainey & McKaskey, 2015). Hence, I only report projected lethal doses for datasets where a sigmoid dose-response was achieved (e.g. the egg life stage) and/or where samples numbers are $n \geq 100$ (e.g. the commercial scenario experiment). These projected doses should still be read with caution.

4.3 Results

4.3.1 Exposed insect rangefinders

72-hour fumigation

There was no significant difference between life stage, dose, or temperature for the rangefinder fumigations (0-400 ppm) with a duration of 72 hours (Appendix: Table 1). Under these conditions, 100% mortality was achieved for eggs, with a calculated LD_{99} (± SE) of 266.7 ± 93.7 ppm for 10℃, and 260.3 ± 115.0 ppm for 20℃ (Fig. 6A & B).

Table 2. 72-hour rangefinder fumigation parameters with life stage, temperature, and dose range.

Life stage	Temp (℃)	Dose-range (ppm)
Eggs	10	0-350
	20	0-400
Larvae	10	0-200
	20	0-160
Pupae	10	0-350
Adults	10	0-350
	20	0-160

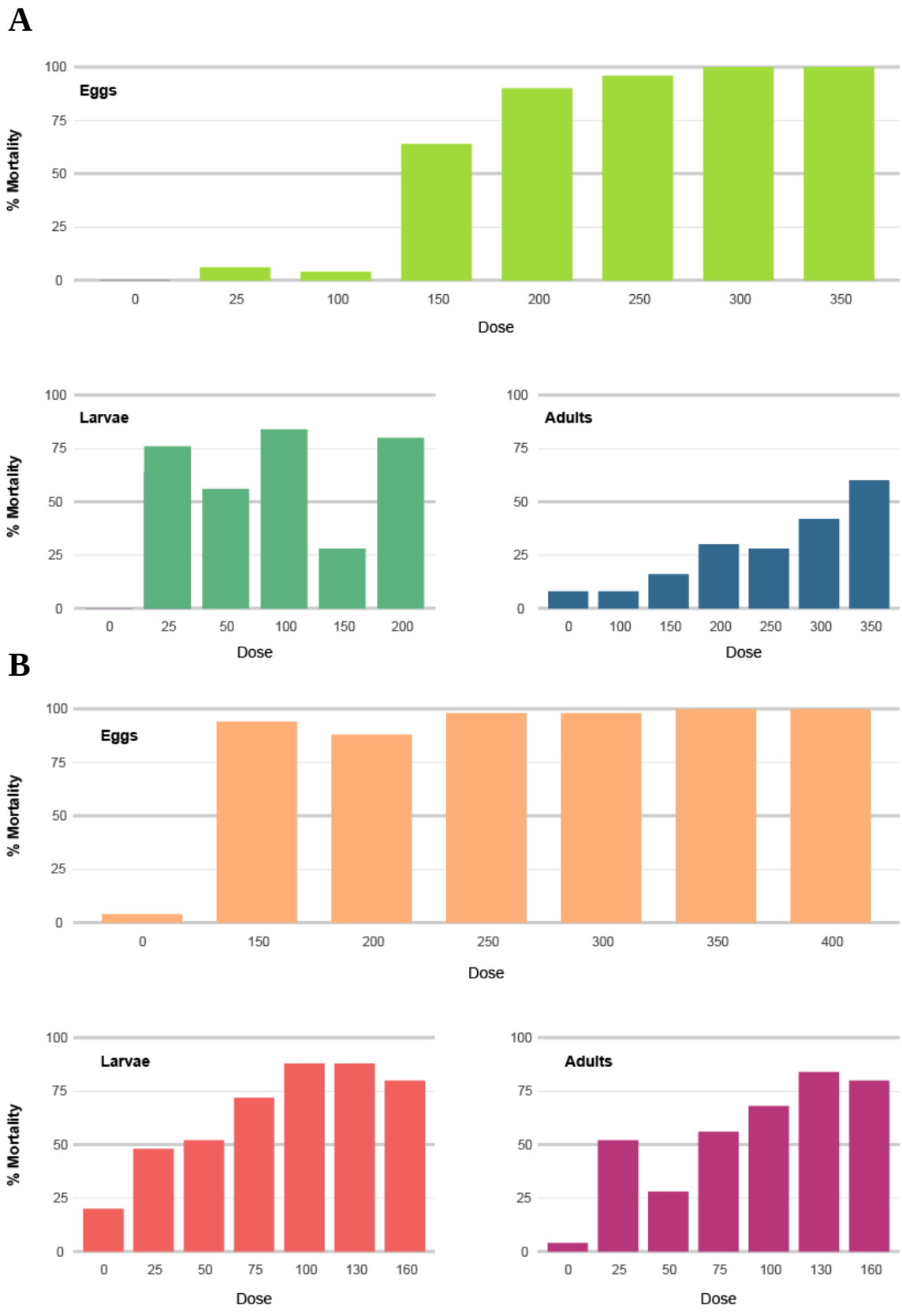

Fig. 6 A) *Hylurgus ligniperda* (F.) eggs (n = 50), larvae (n = 25) and adult (n = 25) percentage mortality at 10°C and **B)** *Hylurgus ligniperda* (F.) eggs (n = 50), larvae (n = 25) and adult (n = 25) percentage mortality at 20°C from exposure to a range of phosphine (PH_3) doses over a 72-hour fumigation period.

In the 72-hour fumigations mortality from PH_3 exposure for larvae and adults was higher at 20℃ as compared to 10℃ (Fig. 6A & B). A higher total (± SE_p) mortality was achieved for larvae at 20℃ with the lower dose of 160 ppm (80 ± 8.0%) as compared to 10℃ (72 ± 9.0%) with a higher dose of 250 ppm (Fig. 6A & B). Similarly, the highest mortality achieved for adults at 10℃ was 60 ± 6.9% at 350 ppm, whereas at 20℃ 84 ± 7.3% mortality was achieved at 130 ppm (Fig. 6A & B). Pupae had the lowest mortality over the dose range (0-350 ppm), with only 28 ± 9.0% mortality recorded for a dose of 350 ppm at 10℃ (Appendix: Fig. 1, Table 1).

120 to 240-hour duration rangefinders

The highest mortality for larvae (n = 50) in the 240-hour rangefinder fumigation was 86 ± 4.9% (100 ppm), and adult (n = 50) mortality was 100% across all doses (25-160 ppm) except for the control in the 240-hour rangefinder fumigation. However, the percentage mortality differences noted above between these two life stages were not statistically different in the GLM model output (Appendix: Table 2). For the 120-hour pupae fumigation (pupae n = 25) the highest mortality achieved of 56 ± 9.9% (1,200 ppm).

Table 3. 120-240-hour rangefinder fumigation parameters with life stage, temperature, duration, and dose range.

Life stage	Temp (℃)	Duration (h)	Dose-range (ppm)
Larvae	10	240	0-100
Pupae	10	120	0-1400
Adults	10	240	25-100

240-hour duration pupae only rangefinders

There was a clear positive relationship between an increase in dose and pupae mortality (r_s = 0.89, P = 4.5e-08). Dose was also a significant factor ($P < 0.05$, 95% CI = {1.00, 1.01}, Appendix: Table 3) between the three exposed pupae rangefinder replicates (fumigation parameters: 240h duration, dose range 0-1400, 10℃), with a mortality (± SE_p) of 98 ± 2.0% achieved for both replicate B and C at the maximum dose of 1,400 ppm over 72 hours (Fig. 7). Replicate A achieved 92 ± 3.8% mortality at the maximum dose of 1,200 ppm (Fig. 7).

Table 4. 240-hour pupae fumigation parameters with rangefinder replicate, temperature, and dose range.

Replicate	Temp (℃)	Dose-range (ppm)
A	10	0-1200
B	10	0-1400
C	10	0-1400

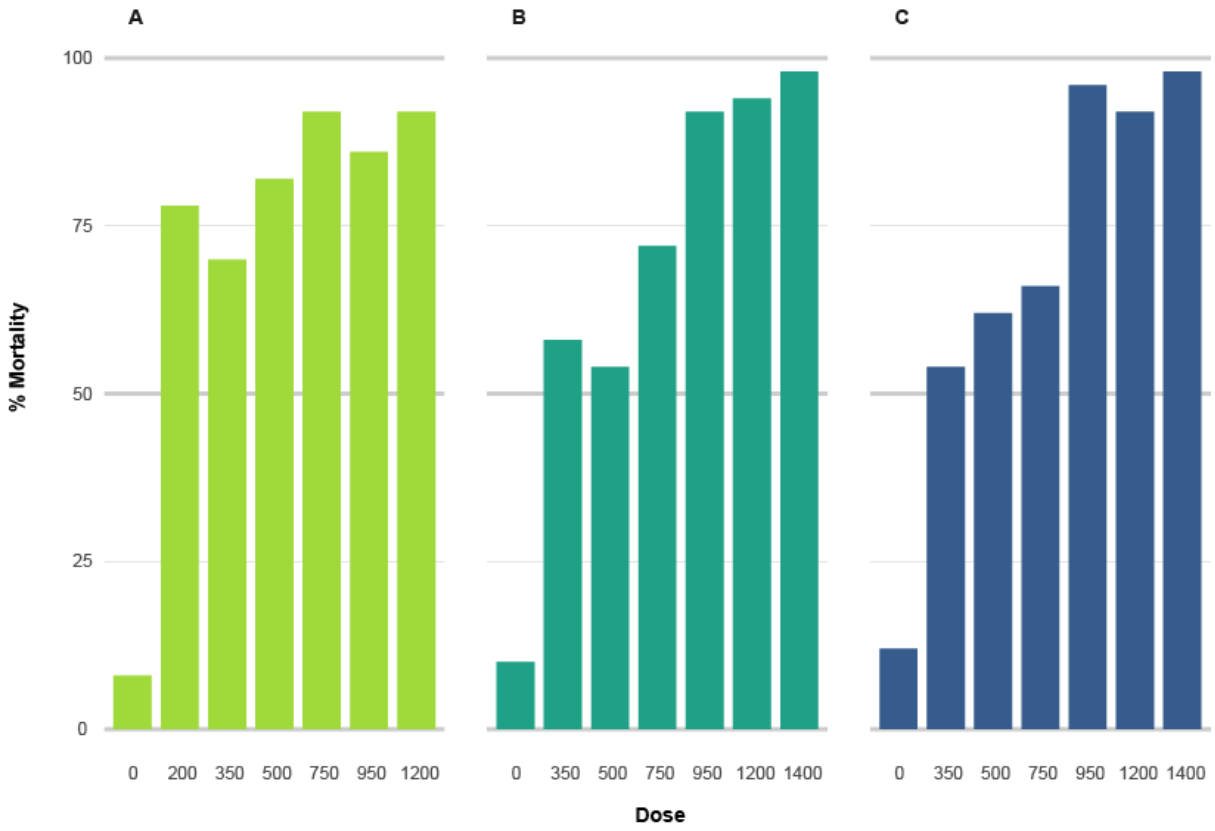

Fig. 7 *Hylurgus ligniperda* (F.) pupae (n = 50) percentage mortality at 10°C across three replicates (A, B and C) from exposure to a range of phosphine (PH_3) doses (ppm) over a 240-hour fumigation period.

4.3.2 In-log insect rangefinders: narrow & extended dose range

The mortality response to PH_3 from larvae was significantly higher compared to pupae in the in-log rangefinder fumigations (*P < 0.05*, 95% CI = {1.16, 6.27}, Table 5 & Appendix: Table 4). 100% mortality was achieved for larvae at 100 ppm (n = 17) for the narrow dose rangefinder (dose range 0-300 ppm), and 100% mortality was also achieved in the extended dose rangefinder (dose range 0-1,400) at 700 ppm (n = 27) (Fig. 8).

Table 5. Pairwise comparison of different *Hylurgus ligniperda* (F.) life stages to phosphine (PH_3) from in-log rangefinder fumigations over 240 hours at 10°C using estimated marginal means with Tukey adjusted *P* values

Life stage contrast	***Estimate***	***SE***	***df***	***z.ratio***	***P***
Adult – larvae	-0.992	0.43	Inf	-2.307	ns
Adult – pupae	*0.189*	*0.434*	Inf	*0.436*	ns
Larvae - pupae	1.182	0.477	Inf	2.476	*****

ns, not significant, * P < 0.05

A phosphine dose between 0-300 ppm achieved a maximum of 72 ± 10.6% (n = 18) mortality (± SE_p) for pupae at 250 ppm, whereas a higher dose of PH_3 (range 0-1,400 ppm) achieved 49 ± 5.9% (n = 73) mortality in this life stage at 1400 ppm (Fig. 8). Similarly, for adults, a higher mortality was achieved with lower concentrations of PH_3, 84 ± 1.9% (n = 315) at 500 ppm than when the dose was increased, 43 ± 4.7% (n = 111) at 1400 ppm (Fig. 8).

The total sample sizes for all life stages were lower in the narrow dose rangefinders compared to the extended dose rangefinders (larvae narrow dose n = 314 vs extended dose 900, pupae narrow dose n = 125 vs extended dose 1082, and adults narrow dose n = 1242 vs extended dose 2139) potentially skewing results.

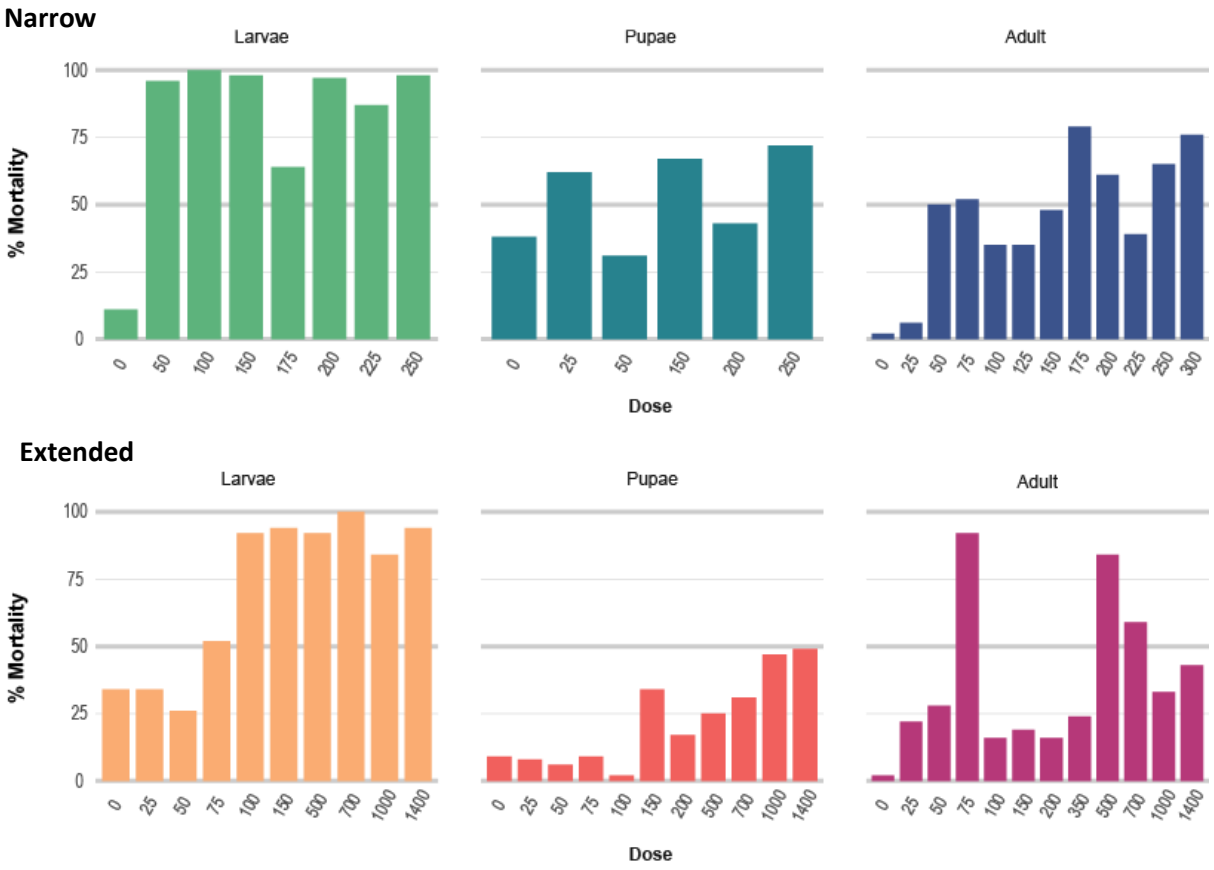

Fig. 8 *Hylurgus ligniperda* (F.) larvae, pupae, and adult percentage mortality from exposure to phosphine at 10°C across two in-log rangefinder fumigations: narrow dose range (0-400ppm) **(top graphic)** and extended dose range (0-1400ppm) **(bottom graphic)** over a 240-hour fumigation duration.

4.3.3 Commercial scenario

There was no significant difference between the mortality of the insects fumigated with PH_3 in the no top-up group (120-hour fumigation, 2 g/m^3 PH_3 with no top-up) and those in the top-up group (commercial schedule - 240-hour fumigation with 2 g/m^3 + 1.5 g/m^3 top-up at 120 hours) nor was there any significant difference between the replicates for each group (Appendix: Table 5). As expected, there was a significant difference from the response of PH_3 dosed insects versus the control insects ($P < 0.001$, 95% CI = {1.00, 1.00}; Appendix: Table 5).

The no top-up group consistently produced mortality responses over 90% (91, 98 and 97%) across all three replicates whereas the mortality responses for the top-up group was lower (77, 94 and 78%). The highest mortality (± SE_p) response for the no top-up group was 98 ± 0.7% (n

= 443), and 94 ± 1.4% (n = 270) for the top-up group. Neither group achieved LD_{99} (Fig. 9; for projected LD_{99} see Appendix: Fig. 3).

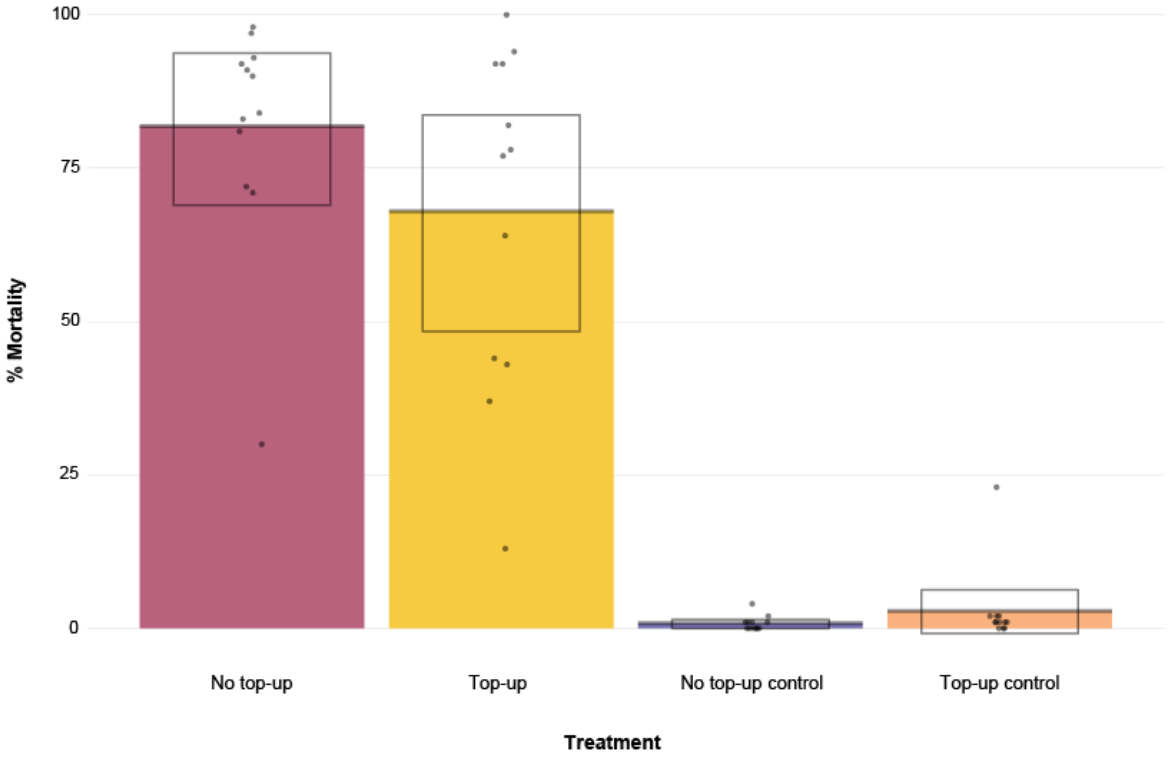

Fig. 9 *Hylurgus ligniperda* (F.) adult average percentage mortality from phosphine (PH_3) exposure at 20℃ over either 120 or 240 hours for the no top-up group and the top-up group with their respective control groups. The average line is represented at the top of the bars, clear overlaid rectangles represent 95% confidence intervals, and grey dots represent raw data points.

The calculated CT product (ppm/hour) for the no top-up group average (± SE) was 63,988 ± 6, 358, and for the top-up group was 131,992 ± 18, 090. Assessment of PH_3 gas readings for both groups from each day show that the no top-up group dropped below 200 ppm (the stipulated minimum threshold in the commercial schedule) by 96 hours (Fig. 10), and the top-up group dropped below 200 ppm between 216-240 hours post 120 hours top-up.

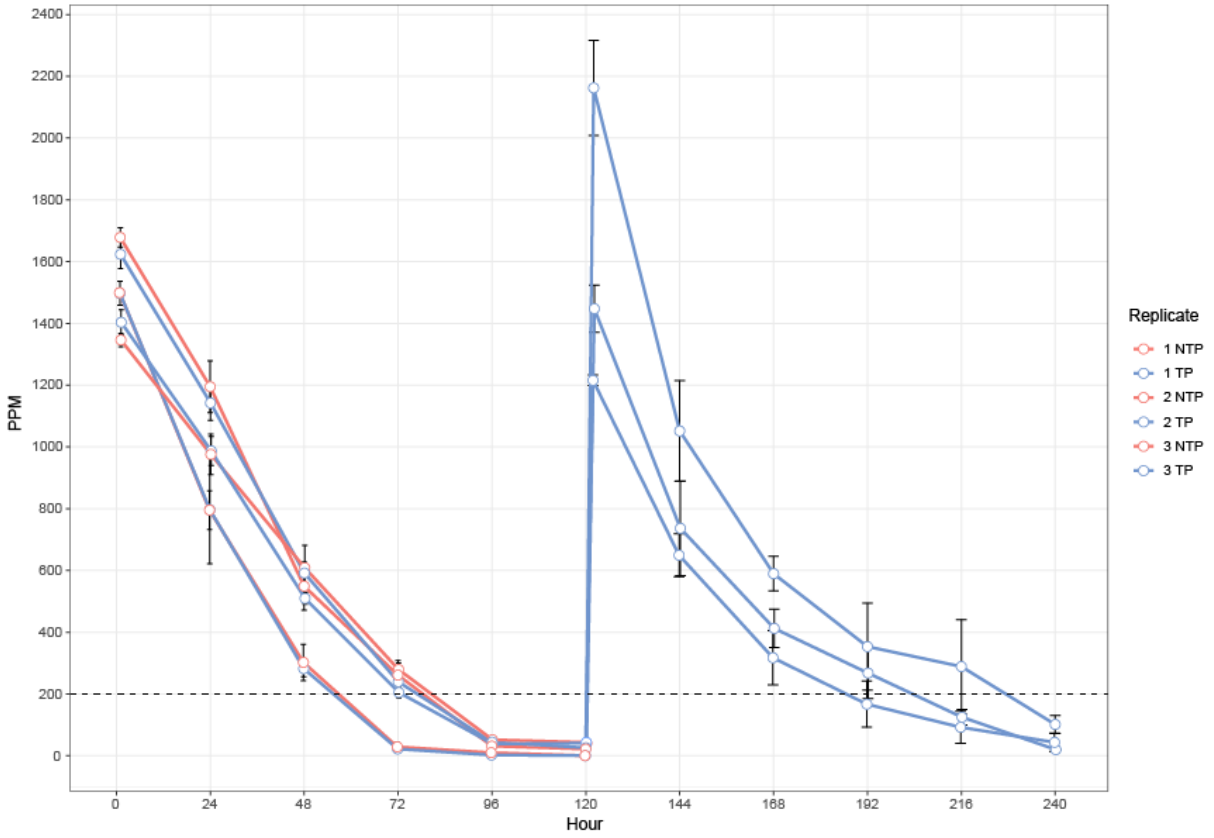

Fig. 10 Mean phosphine (PH_3) (± SE) gas reading in parts per million (ppm) for each replicate within each group every 24 hours. No top-up group (NTP) = replicates 1, 2, and 3. Top-up group (TP) = replicates 1, 2, 3. Dashed line at 200 ppm represents minimum required phosphine level in the commercial schedule.

4.4 Discussion

Hylurgus ligniperda showed widely varying responses to PH_3 fumigation depending on life stage, temperature, dose, and duration. In terms of individual life stage tolerance to PH_3, from least tolerant to most tolerant were - eggs, larvae, adults, and pupae. Eggs were the only life stage that achieved 100% mortality at both 10 and 20°C under the conditions tested. (Fig. 6A & B). Temperature had a measurable effect on the mortality response of larvae and adults to PH_3 in the exposed insect fumigations. Although concentration appeared important in the response from the insects, higher doses did not always indicate higher mortality when duration and temperature were the same, which was seen predominantly under 10°C temperature treatments. This was observed in the exposed insect experiments (Fig. 6A & B) as well as in-log narrow and extended dose experiments, where it was particularly true for pupae and adult *H. ligniperda* (Fig. 8). Duration was particularly important for the exposed pupae response, with LD_{99} nearly achieved for exposed pupae fumigations over 240 hours (Fig. 7), whereas the exposed pupae fumigations of 72 and 120 hours barely achieved LD_{50} (Appendix: Fig. 1).

Contrary to what was found in previous unpublished studies of *H. ligniperda*, the egg life stage was the least tolerant to PH_3 in this study, rather than the previously reported most tolerant stage (Armstrong, Brash, Waddell, et al., 2014). LD_{99} was readily achieved at both 10 and 20°C over a 72-hour fumigation at 300 ppm and 350 ppm respectively. Eggs in the control treatment had good survival in both experiments (> 92% survival). In general terms, insect eggs are usually considered the most tolerant to fumigants, thus tend to require higher doses and longer exposure times (Athanassiou et al., 2012; Gautam et al., 2014). Eggs do not have a tracheal system with spiracles for gas exchange as do other insect life stages. Although there are small openings across the surface of the egg (aeropyles and micropyles) the diffusion of a fumigant through these minuscule openings is unknown, and thought to be one of the reasons why eggs typically have a higher tolerance to fumigants than other life stages (Gautam et al., 2014). However, tolerance of fumigants by insect eggs is very much species-specific, in part due to species-specific egg morphology differentiation (Gautam et al., 2014). For instance, no aeroplyes or microplyes were found on the PH_3 resistant red flour beetle (*Tribolium castaneum*) when compared to the much less resistant Indian-meal moth (*Plodia interpunctella*), which had fourteen aeroplyes (Gautam et al., 2015). The morphology of *H. ligniperda* eggs could be a reason why they are more susceptible to PH_3 compared to other insect pests, but this is currently unknown.

Larvae were the second least tolerant life stage to PH_3 in my experiments. However, in the 72-hour exposed rangefinder experiments larvae and adults responded nearly equally to PH_3 at both 10 and 20°C. (Fig. 6A & B). When fumigation duration for the exposed insect rangefinders was increased to 240 hours, LD_{99} was not achieved for larvae whereas adults reached 100% mortality at the lowest dose of 25 ppm, suggesting that larvae were more tolerant to PH_3 than adult *H. ligniperda*. Interestingly, the subsequent in-log experiments showed that adults were more tolerant in this situation than larvae by ~ 50%, leading to the conclusion that adults are more tolerant than larvae in simulated real-life conditions.

Pupae appeared to be the most tolerant life stage to PH_3, and this is congruent with other studies (Nayak et al., 2020), including earlier unpublished PH_3 studies (Armstrong, Brash, Waddell, et al., 2014). In an analysis of PH_3 tolerance against thirteen different species of stored product beetles, pupae and eggs were considered the most tolerant life stages for twelve of the species (Hole et al., 1976). Similarly, red flour beetle pupae (*Tribolium castaneum*) have been found to be the most tolerant to PH_3 (Manivannan, 2015). Likely the uptake of PH_3 is proportional to the respiration rate of the insect (Chaudhry, 1997), hence a lower respiration rate in the quiescent pupae could account for a lower uptake of the fumigant (see Pimentel et al., 2007). In modified atmosphere studies, pupae have also been found to be more tolerant to hypoxic (low O_2) conditions when compared other life stages within their species; this is thought to be a result of reduced metabolism (Kumar et al., 2017; Mbata et al., 2000). This is congruent with findings within this thesis, where *H. ligniperda* pupae were found to be the most tolerant to low O_2 atmosphere's when compared to larvae and adults (See Chapter 3: Metabolic response to hypoxia and hyperoxia in an endophagous bark beetle, *Hylurgus ligniperda* (F.)).

The higher temperature of 20°C was linked with higher mortality responses from *H. ligniperda* at lower PH_3 doses when compared to higher PH_3 doses at the lower temperature of 10°C. For instance, in the 72-hour fumigation with 100 ppm of PH_3, the mortality of adult *H. ligniperda* was only 8% at 10°C, however, at 20°C mortality was 68%. Temperature plays an important role in PH_3 effectiveness, due to how temperature influences insect metabolism, and thus, insect respiration (Chaudhry, 1997). For instance, temperature was the most preponderant variable compared to humidity and concentration when assessing the efficacy of PH_3 against *Liposcelis bostrychophila*, with higher temperatures leading to increased efficacy (Nayak & Collins, 2008). Further, many of the reports of PH_3 efficacy against bark beetles were undertaken at relatively warm temperatures (i.e. 15-18°C Zhang et al., 2004; 20°C Armstrong, Brash, Waddell, et al., 2014) which is potentially why high mortality was observed. In this thesis,

insects that were exposed to 20°C during respiration studies, showed a significantly higher respiration rate across all the life stages when compared to a lower temperature of 10°C (See Chapter 3: Metabolic response to hypoxia and hyperoxia in an endophagous bark beetle, *Hylurgus ligniperda* (F.)).

There was a clear dose-response relationship for the exposed pupae fumigations. LD_{99} was nearly achieved with a 240-hour fumigation duration and mortality increased consistently with dose (Fig. 7). This was not seen for pupae in-log with the same duration and similar dose range, potentially as a result of PH_3 sorption into the logs, along with other factors introduced by the logs, such as atmospheric modification through carbon dioxide increase and O_2 decrease (Svedberg et al., 2009). PH_3 doses remained relatively consistent throughout the exposed pupae experiments (Appendix: Fig. 2) but not for experiments with insects within the logs (Fig. 10). The variability seen throughout the PH_3 headspace samples in is likely a result of PH_3 sorption into either insect diet in the case of exposed insects or pine logs in the case of in-log experiments. Up to 100% sorption has been noted in moist products over the course of a seven day fumigation (Reddy et al., 2007). This in conjunction with low sample numbers is likely an explanation for the high variability seen in the headspace samples; it should be noted that the minimum prescribed dose of over 200 ppm was not maintained for the entire duration of the commercial scenario experiment, as stipulated by the commercial schedule (Ministry for Primary Industries, 2018b). Within approximately four days of the initial dose, and top-up dose, both groups had below 200 ppm in the headspace atmosphere of the chambers (Fig. 10).

For in-log experiments the implementation of higher dose ranges did not consistently increase mortality. For instance, in the commercial scenario experiment, the full commercial schedule (a total of 3.5 g/m^3 of PH_3 applied) appeared less effective in terms of percentage mortality than the no top-up experiment that had only 2 g/m^3 applied. Mortality was also variable in the in-log experiments; there were several instances of higher mortality at lower doses, and lower mortality at higher doses during the same experiment. However, because of the low insect numbers particularly in the narrow dose rangefinders along with the other variables of low temperature, and the use of insects in logs, the results are unclear.

There are several issues that could cause increasing doses of phosphine to be less effective. For instance, it is thought that high doses of PH_3 over short periods can trigger a narcotic state in some insect species, leading to less fumigant uptake (Winks, 1985; Cao & Wang, 2001). This has been shown to have a protective effect for insects undergoing fumigation and could

potentially be the reason why higher mortality was often observed at lower doses in this study. A potential explanation could be hormesis, which is the biphasic relationship seen when an organism is subjected to a sublethal dose of a chemical (or physical stressor) prior to a lethal dose, leading to an inhibited response to the lethal dose (Ma et al., 2017).

The use of concentration x time (CT) as a measure of effectiveness or as an indication of total exposure can be misleading, which is especially the case for PH_3 (Winks, 1986) Although very high CT products were achieved in this study, particularly in the commercial scenario experiment, it did not translate to more effective control, with the higher CT product having less efficacy than the lower CT product (Fig. 10).

Duration of fumigation was an important variable in predicting mortality of the insects. In the exposed insect fumigations, 72-hour exposure to PH_3 was insufficient to achieve LD_{99} for the more tolerant life stages, particularly pupae. However, increasing fumigation from 72 hours to 120 hours, and then 240 hours, nearly doubled the pupa response to PH_3. Further, the exposed 240-hour fumigation of adults achieved 100% mortality at doses as low as 25 ppm at 10°C, whereas at 72 hours the highest mortality achieved was 60% at 350 ppm (Fig. 6A). In terms of PH_3 efficacy, the duration of the exposure period has been found to be one of the most important factors in determining insect control (Chaudhry, 1997; Flinn et al., 2001; Howe, 1973). Longer durations (> 7 days) are also cited as a method to overcome more tolerant life stages. For more resistant insect species, the lethal time to reach 99% mortality can be up to 27 days (Collins et al., 2001). The duration of exposure is thought to be linked to insect respiration, hence lower temperatures warrant longer exposure periods, and under higher temperatures, the duration can be lessened (Collins et al., 2001; Shi et al., 2012).

4.5 Conclusion

The response of *H. ligniperda* to PH_3 is variable, and depends on temperature, duration, life stage and dose. Dose rate of PH_3 appeared less important than duration or an increase to warmer temperatures. PH_3 was most effective at 20°C and fumigations exceeding 120 hours. *H. ligniperda* eggs were the life stage most susceptible to PH_3. Pupae and adults were found to be the most tolerant life stages, which is congruent with other PH_3-treated insect species. Exposed insects were more susceptible to PH_3 than insects within the logs, with 100% mortality nearly achieved for exposed pupae.

In-log fumigations demonstrated that achieving 100% mortality is markedly harder than fumigation of exposed insects, most likely due to variables introduced by the logs themselves within an enclosed space (See Chapter 2. Respiration by unprocessed pine logs creates a severe hypoxic and hypercapnic atmosphere in enclosed spaces). The assessment of the commercial schedule showed that the shorter duration of 120-hours without the top-up was more effective than the 240-hour duration with the top-up. However, PH_3 gas levels were not maintained above 200 ppm throughout this experiment. Potential future work could focus on similar dose ranges as used in this study, with significantly longer durations $\geq$ 20 days, as well as ensuring consistent PH_3 levels throughout the fumigation period.

Chapter 5 - General discussion

5.1 Key findings

The effective disinfestation of wood products for trade is critical to halting the spread of invasive forest insect species (Allen et al., 2017). Currently, invasive insects are being transported inadvertently with wood products (Inward, 2019; Faccoli et al., 2020), despite an increasing awareness of the problem (Pyšek et al., 2020). The most widely used fumigant for wood products, methyl bromide (MB), is due to be phased out globally, so finding effective replacement treatments is a pressing need (Holmes et al., 2020). The current New Zealand deadline for discontinuing the release of MB into the atmosphere is February 2022 (Environmental Protection Agency, 2021), yet there is still a lack of suitable replacement tools. Phosphine (PH_3) has been in use for several decades in New Zealand to disinfest whole unprocessed pine logs during shipment, as an alternative to MB (Ministry for Primary Industries, 2018b). Little was known about the unique abiotic and biotic variables resulting from the respiration of whole pine logs within relatively airtight ship holds. Further, the physiology of the target pest, bark beetles, or how their physiology may influence the effectiveness of disinfestation treatments in a real-world setting within ships holds was largely unknown.

In this thesis, I focused on three fundamental knowledge gaps relating to the use of PH_3 for the disinfestation of pine logs in transit. Firstly, I investigated the respiration of cut whole radiata pine logs (*Pinus radiata* D.Don) and how this may influence the atmosphere within an enclosed space. There was little published information about these types of products (Chapter 2). I then sought to understand the gas exchange patterns and respiratory tolerances of *Hylurgus ligniperda* (F.) as a model organism for easily transported invasive bark beetles (Chapter 3). The fundamental physiology of endophagous insects, particularly bark beetles, is poorly explored in the literature. Finally, I directly tested the efficacy of PH_3 against *H. ligniperda* using a series of rangefinder fumigation experiments (Chapter 4). The results of this study provide a foundation for assessing PH_3 efficacy in a wood disinfestation setting (Fig. 1).

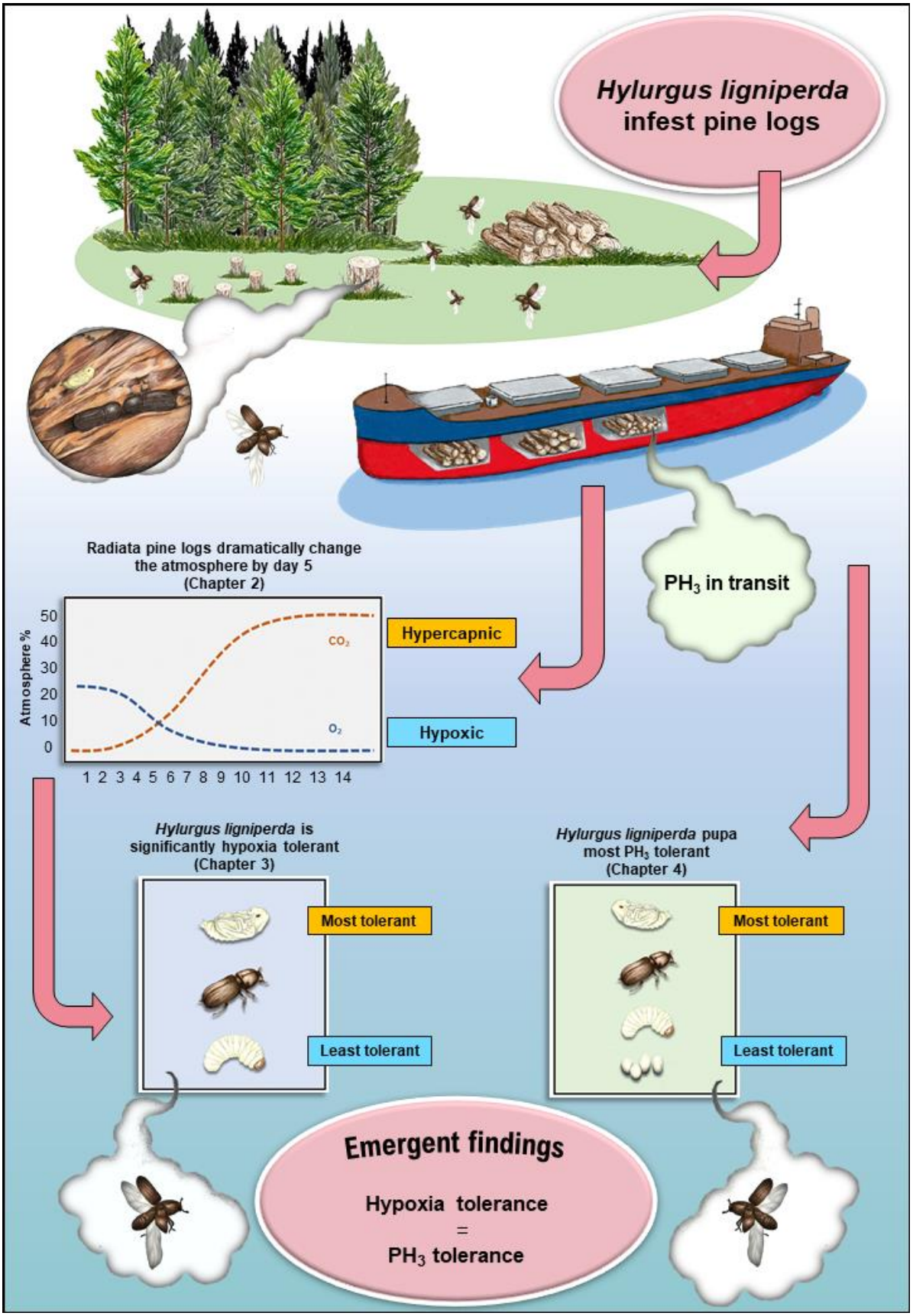

Fig. 1 Summary infographic of the key findings from each data chapter. Pink arrows indicate the flow of ideas and findings throughout the thesis. Illustration, Devitt, 2020.

My main findings in Chapter 2 addressed the atmospheric conditions produced by radiata pine logs within an enclosed chamber; this was as a proxy to on-board log transport conditions. Using a multifactorial design, I sampled carbon dioxide (CO_2) emission, and oxygen (O_2) reduction daily for 14 days from pine logs harvested at four different seasons; austral spring, summer, autumn, and winter at three different temperatures. As predicted, I demonstrated that respiring pine logs dramatically and quickly change the surrounding atmosphere to severely hypoxic (low O_2) and hypercapnic (high CO_2) within days (Fig. 1). Importantly, I also demonstrated that there were strong seasonal differences in the amount and rate of CO_2/O_2 amongst the cut trees. My findings on the rate and amount of CO_2/O_2 present in this study were congruent with unpublished data recorded on board the ship during log transport (Hall et al., 2016), as well as other wood product transport/containment studies (Svedberg et al., 2009; Feng et al., 2015). As expected, higher temperatures (15 – 20°C) brought about a faster increase in CO_2 emission and a decrease in O_2, compared to the lower temperature of 10°C. The main contribution of my research was to quantify the rate of atmospheric changes, as such a rapid and substantive change in conditions is important to consider when developing and evaluating treatment methods.

Information on the respiration of bark beetles is scarce (Gehrken, 1985; Hansen et al., 2011), and to my knowledge, there are no published studies on their potential tolerance to low O_2. Further, differences in respiration throughout ontogeny in insect species are depauperate (Greenlee & Harrison, 2005). I sought to understand the inherent tolerances of *H. ligniperda* to low O_2 across different life stages, as well as their responses to varying levels of O_2 under different temperature and moisture conditions. I used the results from my log respiration study (Chapter 2), to select the O_2 treatment levels in my insect respiration study (Chapter 3). Gaining this fundamental information will allow us to further understand if *H. ligniperda* may have an inherent tolerance to fumigants such as PH_3 that require active uptake. I found that *H. ligniperda* was highly tolerant of hypoxic atmospheres across all three life stages tested: larvae, pupae, and adults. Pupae were the most tolerant to hypoxic atmospheres (Fig. 1). This is congruent with the pupae of other insect species, and likely a result of quiescence in this life stage (Howe, 1973; Manivannan, 2015; Venkidusamy et al., 2018).

In Chapter 4 I assessed the efficacy of PH_3 against *Hylurgus ligniperda* as a model pest species. This study measured the efficacy of PH_3 for achieving 100% mortality across all four life stages of *H. ligniperda*, eggs, larvae, pupae, and adults, at two different temperatures, using various durations. Overall, 100% efficacy was not achieved for all life stages of *H. ligniperda*; pupae

were the most tolerant life stage. Contrary to prior findings (Armstrong, Brash, Waddell, et al., 2014), I found the egg life stage was the most sensitive to PH_3, and the response from eggs to PH_3 was comparable at both 10 and 20°C (Fig. 1). Both temperature and duration of fumigation had a larger impact on efficacy than the dose (Chapter 4). The results from this study are informative of the effectiveness of PH_3 against *H. ligniperda*; although my results should not be viewed as conclusive because of the low number of replicates and the resulting large margin of error.

5.2 Phosphine use in hypoxia tolerant species

The reported interceptions of live insects in New Zealand export pine logs highlight that PH_3 is not always effective against bark beetles in this environment (Zhang et al., 2004; Ministry for Primary Industries, 2015). When examining some of the mechanisms that can make PH_3 less effective, insect resistance has been at the forefront (Holloway et al., 2016; Nayak et al., 2020). However, genetic resistance is highly unlikely to develop in New Zealand populations of bark beetles via the trade in pine logs because beetles are only exposed to PH_3 as they are shipped out of the country. Nevertheless, considering a genetic basis for PH_3 tolerance in bark beetle species like *H. ligniperda*, is important as these beetles could be inherently tolerant to PH_3 because of their ability to tolerate low O_2 environments (Chapter 3).

One of the genetic mechanisms identified in PH_3-resistant insect populations is related to a mutation in the gene responsible for aerobic respiration and energy metabolism in the mitochondria (Carothers, 1989; Schlipalius et al., 2012; Alzahrani & Ebert, 2019a). The downregulation of the dihydrolipoamide dehydrogenase (*dld*) enzyme lowers metabolism via associated metabolic pathways - oxygen phosphorylation (OXPHOS) and the tricarboxylic acid cycle (TCA). A reduction in aerobic respiration by ~ 75% in resistant *Caenorhabditis elegans* strains is thought to be a key factor in protecting them from PH_3. It was found that PH_3 resistant *C. elegans* strains are also resistant to the normally synergistic combination of PH_3 and super-atmospheric O_2 levels (hyperoxia) (Cheng et al., 2003; Zuryn et al., 2008). The reduction in metabolism via genetic mechanisms affords protection against the O_2/PH_3 combination, likely to be due to a decrease in ROS production (Alzahrani & Ebert, 2019b). It is interesting to note that *H. ligniperda* pupae reduced their respiration in response to hyperoxia, rather than increasing it as would be expected (Alzahrani & Ebert, 2019b). This could be because they were already in a similar state of metabolic suppression as seen in resistant species, and the introduction of high O_2 levels elicited a protective response.

The metabolic mechanisms identified in PH_3-resistant insects are akin to the mechanisms seen in insect species that are adapted to hypoxic environments (Pimentel et al., 2007). The ghost moth (*Thitarodes armoricanus*) is adapted to high-altitude environments and survives hypoxia through the suppression of several enzymes in the TCA cycle including *dld*; and the bulk of genes associated with OXPHOS are also suppressed (Rao et al., 2019). Similarly, the hypoxia-resistant cowpea bruchid (*Callosobruchus maculatus*) showed a down-regulation in the genes that encode TCA cycle enzymes (Ahn et al., 2013). Although there is limited evidence of bark beetle adaptions to hypoxia, genetic or otherwise, it seems logical that insects that have an inherent ability to survive in hypoxic conditions may also have an intrinsic resistance to PH_3 toxicity. Different life stages of *H. ligniperda* had comparable tolerances to both hypoxia and PH_3 (Fig. 1); the relative tolerance to hypoxia and PH_3 of each life stage, from most to least tolerant was the same. A significant correlation between hypoxia and PH_3 has been observed in several PH_3-resistant grain pests (Pimentel et al., 2007). Consequently, the PH_3 efficacy data (Chapter 4), and the metabolic data in Chapter 3 are consistent with *H. ligniperda* being inherently tolerant to PH_3. The degree of tolerance shown by *H. ligniperda* adults in the commercial scenario experiment (Chapter 4) was similar to resistance levels seen in a highly PH_3-resistant strain of the rusty grain beetle (*Cryptolestes ferrugineus*) (Kaur & Nayak, 2015). Considering hypoxia resistant species, and/or species that are likely to be hypoxia resistant, as also potentially inherently tolerant to fumigants such as PH_3 is likely to provide insights into how to develop effective control tools for insects.

5.3 Improving insect biosecurity

My thesis demonstrates how an understanding of the fundamental physiology of plants and insects can make an important contribution to insect biosecurity. Respiration by pine logs in the enclosed space, simulating a ship's hold, rapidly changed the atmosphere to severely hypoxic and hypercapnic conditions. Some bark beetles such as *H. ligniperda* are resistant to hypoxia, and this may partly explain why they are also tolerant to fumigants such as PH_3. Furthermore, it suggests that several treatment options that are already utilised for PH_3-resistant pest insect species could be applicable to *H. ligniperda* and other bark beetles.

It is apparent that PH_3 efficacy in insect control is highly dependent on insect metabolism because it requires active respiratory uptake (Pimentel et al., 2007). Any modification to insect metabolism/gas exchange, whether that be from biological or abiotic factors, will directly impact PH_3 uptake (e.g. temperature, Nayak & Collins, 2008; hypoxia, Kashi, 1981; genetic

metabolic resistance, Schlipalius et al., 2012). Factors that decrease insect metabolism should be avoided or mitigated where possible. For example, in Australia PH_3 use is not recommended at temperatures below 15°C (Warrick, 2011; Department of Agriculture Water and the Environment, 2019), because low temperature reduces the efficacy of PH_3 (Chaudhry, 1997). The average winter temperature recorded in-hold during PH_3 fumigation of logs from New Zealand to China ranged from 7.6 – 12.3°C (Hall et al., 2016). PH_3 fumigation begins while the ship is on shore in New Zealand, so the majority of the fumigation has occurred before entering tropical temperatures (Hall et al., 2016). This suggests fumigation during winter months on this route is likely to result in increased insect survival. Increasing the length of exposure to PH_3 may counteract the depressed metabolic effect of low winter temperatures, although research on the PH_3 resistant *Liposcelis bostrychophila* indicates more than 19-days of exposure is needed at 15°C (Kaur & Nayak, 2015).

Hypoxic in-hold atmospheres are likely to decrease insect respiration and PH_3 uptake (Pimentel et al., 2007; Kaur & Nayak, 2015). The amount and rate of decrease of available O_2 will not only be dependent on the hold's integrity, and ambient temperatures, but also on when the pine logs were harvested (Chapter 2). If logs are transported from cool to warm temperatures during transit, then it is likely that hypoxia levels will be higher than if logs were harvested in mild-warm temperatures and transported to warmer temperatures (Chapter 2). Accounting for harvest season and changes in the respiration rate of logs may improve disinfestation practices.

An increase in treatment time seems the most logical approach to allow for resistance of pests to PH_3 (Daglish et al., 2002; Collins et al., 2005; Kaur & Nayak, 2015; Nayak et al., 2020; Chapter 4). Currently, in-transit logs in New Zealand are fumigated for 9 - 15 days (Ministry for Primary Industries, 2018b). The sea voyage from New Zealand to China takes 20 – 30 days (Champion Freight, 2020). If the duration of PH_3 treatment can be extended to ≥ 20 days, this would increase the exposure period of the insects to PH_3 and potentially mitigate lower temperatures (Kaur & Nayak, 2015). Alternatively, if the start of treatment was delayed until the ship reaches warmer temperatures (ideally the ship's hold at 20°C), this might increase the efficacy of PH_3 treatment without having to extend the duration. An increase in PH_3 duration would incur additional costs as there would need to be additional PH_3 top-ups to maintain in-hold levels. However, extending the commercial fumigation period could occur at a lower concentration than currently used, enabling less PH_3 to be used overall; Chapter 4 highlighted that duration was significantly more important for control outcomes than dose.

An increase in O_2 has been shown to increase the efficacy of PH_3 in several efficacy trials (Liu, 2011; Liu et al., 2015; Alzahrani & Ebert, 2019b), with the caveat that this may not be as efficacious in PH_3 resistant species (Alzahrani & Ebert, 2019b). The feasibility of introducing super-atmospheric O_2 to a ship's hold is unknown, and this may also create additional risk factors for fumigators and ship workers if this increases the risk of explosion/flammability (Ryan & Lima, 2014). CO_2 levels recorded within ships holds were relatively high (Hall et al., 2016, ~7%, and Chapter 2). There is evidence that CO_2 can work synergistically with PH_3 on resistant insect pests (Constantin et al., 2020). This may be due to CO_2 increasing insect respiration, hence increasing uptake (Constantin et al., 2020). Further research is required to determine if the levels currently present in-hold are potentially already working synergistically with PH_3.

5.4 Future research

Further research into bark beetle respiration and their respiratory tolerances is needed. Chapter 3 highlighted the lack of research on these types of species, and endophagic insects in general (Pincebourde & Casas, 2016). Since bark beetles are considered to be some of the most invasive species (Brockerhoff & Liebhold, 2017), and are easily moved to new locations (Allen et al., 2017), additional research on their physiology is warranted. Further to this, investigating the evolutionary factors of hypoxia resistance, and whether there is a link between the mechanisms of hypoxia resistance and PH_3 resistance would be a compelling potential correlation to investigate.

A clear dose-response curve for *H. ligniperda* was not achieved for the majority of life stages in this study (Chapter 4). The abiotic conditions introduced by the pine logs during in-log fumigations further confounded the issue of finding a dose-response curve. Establishing a replicable dose-response curve for *H. ligniperda* would be highly beneficial going forward, however, the abiotic factors of the insect's habitat (i.e. within cambium of pine logs) must be incorporated to be helpful in real world applications. A quicker avenue to explore potential PH_3 tolerance in *H. ligniperda* is rapid knockdown bioassay tests for PH_3 resistance (Food and Agriculture Organization, 1975; Nayak et al., 2013; Cato et al., 2019). Rapid knockdown tests enable the researcher to quickly establish the inherent tolerance to PH_3 *ex-situ*, which would save time and can be applied to any insect species. In hindsight, this would have been an easy experiment to include and could offer further insight into *H. ligniperda*'s PH_3 tolerance. By extension, this method may also be a way to disentangle the insect's response to PH_3 from the

abiotic conditions presented by residing within pine logs. Additionally, the knockdown tests could be used for other bark beetle species that are pests associated with radiata pine logs e.g. *Hylastes ater*, *Arophalus ferus* (Brockerhoff et al., 2006).

Ultimately a seasonally repeated *in-situ* experiment with designated bark beetle infested pine logs in-hold would be the gold standard. In theory, this would definitively answer the question as to whether *H. ligniperda* can survive the current fumigation schedule. Further research is also required to determine if other bark beetle species respond in a similar way. However, deliberately transporting invasive species for scientific purposes may not be ethical, nor practical on such a large scale.

5.5 Concluding remarks

Insect control is a complex process, particularly when there are prevailing physiological and abiotic conditions that could be impacting the outcomes. This study highlighted the varied and complex factors present in the real-world situation of pest control for disinfestation purposes. Understanding the fundamental abiotic conditions present during pest control, along with any intrinsic physiology of both the target pest and the commodity will help to achieve better pest control outcomes.

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Appendix

Supplementary information for Chapter 4

Table 1. Mortality of exposed *Hylurgus ligniperda* (F.) eggs, larvae, pupae, and adults fumigated with phosphine at 10 or 20°C over a 72-hour period in contrast to predictor variables of dose, life stage, and temperature. Output from generalised linear model with a probit link, showing risk ratios, standard error (SE), z statistic, and *P* values (at $\alpha = 0.05$). Pseudo- R^2 displayed is Aldrich-Nelson with the Veall-Zimmerman correction.

Predictors	***Risk ratios***	***SE***	***Z***	***P***
(Intercept)	0.19	2.11	-0.77	ns
Dose	0.99	0.01	-0.64	ns
Temperature	0.97	0.13	-0.2	ns
Eggs	2.88	0.99	1.06	ns
Larvae	4.75	0.94	1.66	ns
Pupae	0.39	1.22	-0.77	ns
Dose x Temperature	1.00	0.00	1.33	ns

Observations	48	Pseudo-R^2	0.52
Deviance	7.49	AIC	51.651

ns, not significant

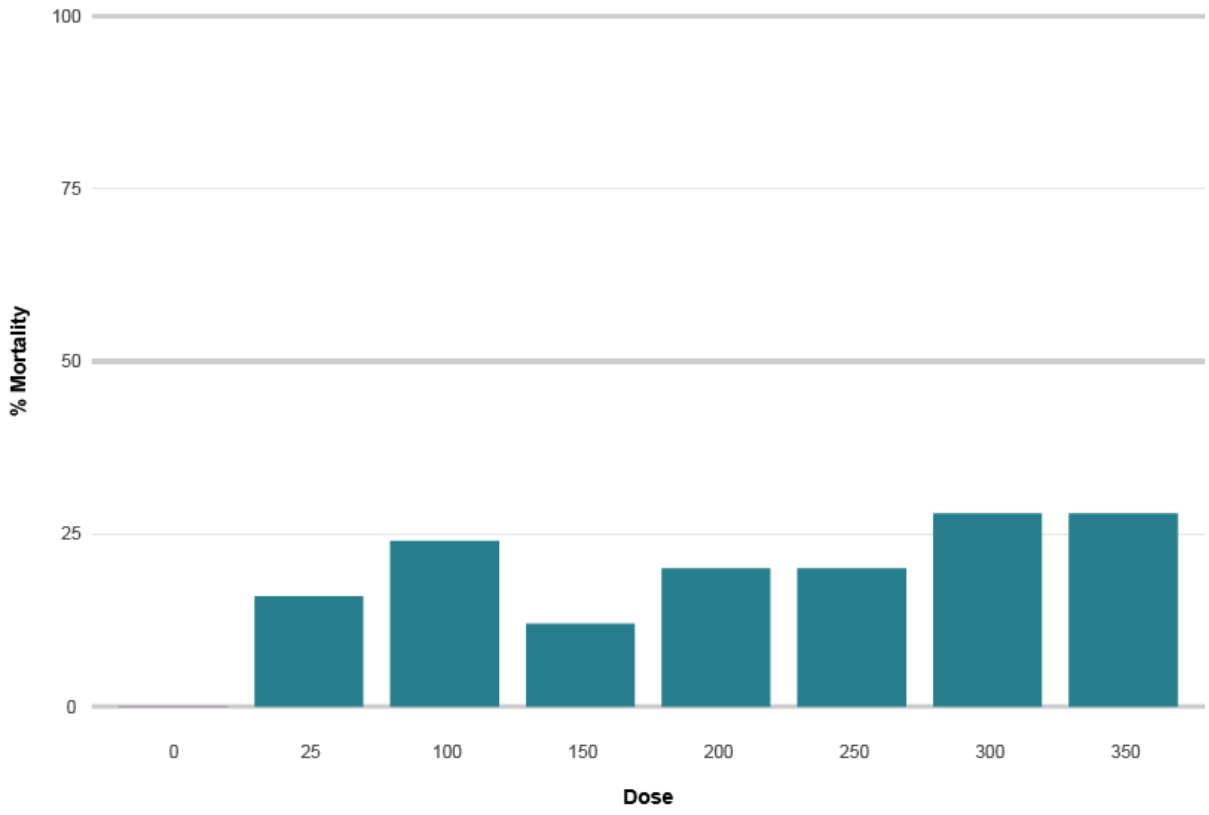

Fig. 1 Exposed *Hylurgus ligniperda* (F.) pupae (n = 25 per dose) percentage mortality at 10°C from exposure to a range of phosphine (PH_3) doses over a 72-hour fumigation period.

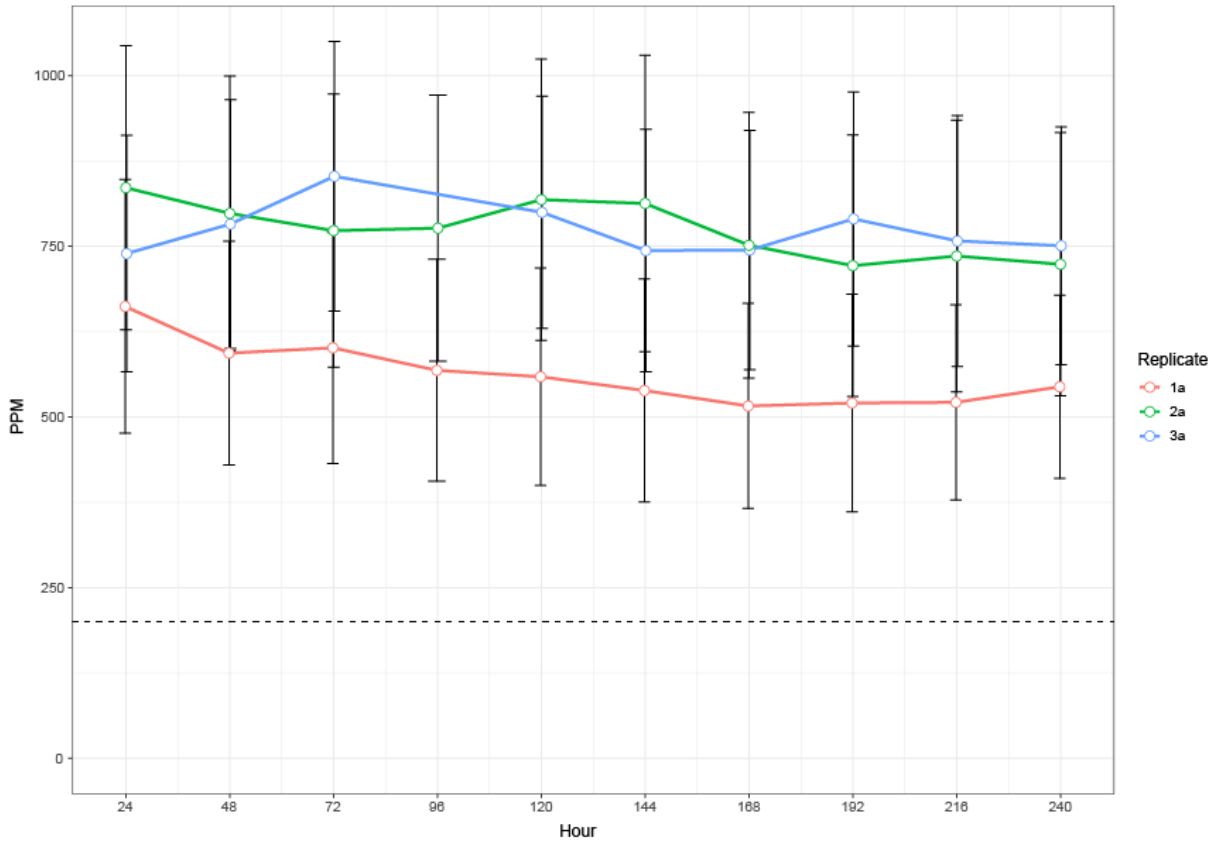

Fig. 2 Mean phosphine (PH_3) (± SE) gas reading in parts per million (ppm) for each replicate in the exposed pupae experiments (dose 0-1200 or 0-1400 ppm), taken every 24 hours up to 240 hours at 10°C. Rep 1 = 1a, rep 2 = 2a, rep 3 = 3a.

Table 2. Mortality of exposed *Hylurgus ligniperda* (F.) larvae and adults fumigated with phosphine at 10°C over 240 hours in contrast to predictor variables of dose and life stage. Output from generalised linear model with a probit link, showing risk ratios, standard error (SE), z statistic, and *P* values (at $\alpha = 0.05$). Pseudo-R^2 displayed is Aldrich-Nelson with the Veall-Zimmerman correction.

Predictors	***Risk ratios***	***SE***	***Z***	***P***
(Intercept)	-2.05	2.89	-0.71	ns
Dose	0.31	72.02	0.00	ns
Larvae	1.81	2.99	0.61	ns
Dose x Life stage	-0.30	72.01	-0.00	ns
Observations	14			
Pseudo-R^2	0.64			
Deviance	1.86			
AIC	14.597			

ns, not significant

Table 3. Mortality of *Hylurgus ligniperda* (F.) pupae fumigated at 10°C over a 240-hour period in contrast to predictor variables of dose and replicate. Output from generalised linear model with a probit link, showing risk ratios, standard error (SE), z statistic, and *P* values (at $\alpha = 0.05$). Pseudo- R^2 displayed is Aldrich-Nelson with the Veall-Zimmerman correction.

Predictors	***Risk ratios***	***SE***	***Z***	***P***
(Intercept)	0.51	1.15	-0.58	ns
Dose	1.00	0.00	2.09	*****
Rep: B	0.43	1.44	-0.59	ns
Rep: C	0.44	1.44	-0.58	ns

Observations	21	Pseudo-R^2	0.57
Deviance	1.31	AIC	19.96

ns, not significant, * P < 0.05

Table 4. Mortality of in-log *Hylurgus ligniperda* (F.) larvae, pupae and adults fumigated in rangefinders with a narrow and extended dose range over a 240-hour period at 10°C in contrast to predictor variables of dose, rangefinder, and life stage. Output from generalised linear model with a probit link, showing risk ratios, standard error (SE), z statistic, and *P* values (at $\alpha = 0.05$). Pseudo-R^2 displayed is Aldrich-Nelson with the Veall-Zimmerman correction.

Predictors	***Risk ratios***	***SE***	***Z***	***P***
(Intercept)	0.86	0.32	-0.45	0.651
Dose	1	0	1.79	0.073
Rangefinder extended	0.5	0.38	-1.8	0.072
Larvae	2.7	0.43	2.31	*****
Pupae	0.83	0.43	-0.44	0.663
Observations	58			
Pseudo-R^2	0.44			
Deviance	14.63			
AIC	70.374			

ns, not significant, * P < 0.05

Table 5. Mortality of *Hylurgus ligniperda* (F.) adults from the commercial scenario experiment fumigated in either group A or group B at 10°C over a 120- or 240-hour period in contrast to predictor variables of dose, group and replicate. Output from generalised linear model with a probit link, showing risk ratios, standard error (SE), z statistic, and *P* values (at $\alpha = 0.05$). Pseudo-R^2 displayed is Aldrich-Nelson with the Veall-Zimmerman correction.

Predictors	***Risk ratios***	***SE***	***Z***	***P***
(Intercept)	0.06	1.42	-1.98	*
Dose	1.00	0.00	2.69	***
Group B	1.45	1.74	0.21	ns
Group B rep 1	1.59	0.85	0.55	ns
Group A rep 2	2.02	1.04	0.67	ns
Group B rep 2	2.50	0.88	1.04	ns
Group A rep 3	1.32	0.96	0.29	ns
Dose x Group	1.00	0.00	-0.60	ns
Observations	48			
Pseudo-R^2	0.84			
Deviance	5.92			
AIC	35.619			

ns, not significant, * P < 0.05 ** P < 0.01 *** P < 0.001

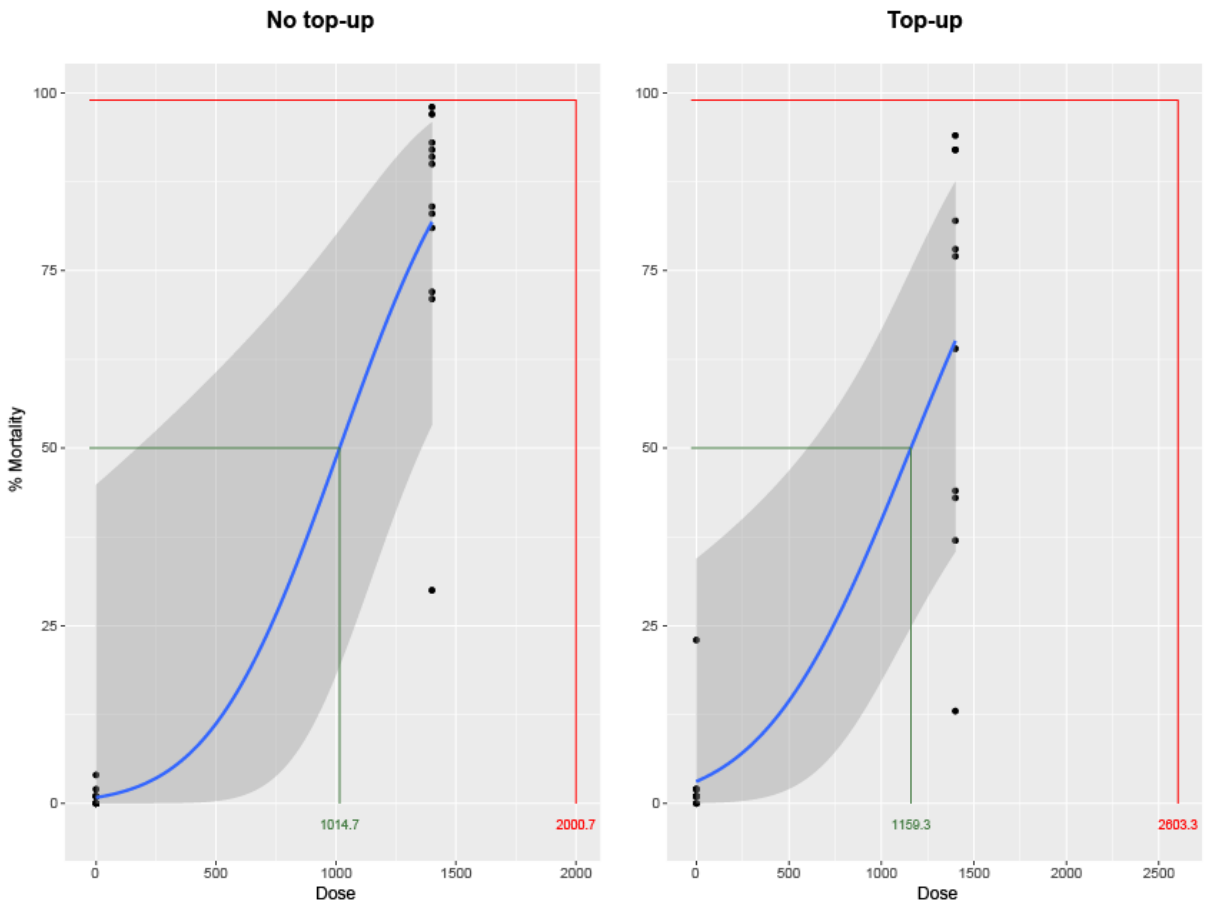

Fig. 3 *Hylurgus ligniperda* (F.) adult percentage mortality from phosphine (PH_3) exposure at 10°C over either 120 or 240 hours for the no top-up group and the top-up group respectively. The blue line indicates the fitted probit regression line, shaded dark grey area indicates 95% confidence intervals, black dots represent the raw data points and the green and red lines are the probability estimated LD_{50} and LD_{99}, respectively.