# Resistance of *Rhyzopertha dominica* (Coleoptera: Bostrichidae) to phosphine fumigation; geographic variation, high dose treatments and rapid assay assessment

by

Edwin Afful

B.S., University of Ghana, 2007
M.S., University of Ghana, 2010

AN ABSTRACT OF A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2018

# Abstract

The emergence of heritable high-level resistance to the fumigant gas phosphine in stored-product insects is of grave concern to many grain growing countries around the world. The research reported in this dissertation was designed to determine the presence of phosphine resistance in 34 field collected populations of *Rhyzopertha dominica* (F.) from the United States and Canada, the potential to control resistant *R. dominica* populations utilizing high dose and longer exposure time strategies, and to develop a rapid assay for phosphine resistance detection. Using a discriminatory dose assay called the FAO number 16 method, adult *R. dominica* were sampled and subjected to a phosphine dose of 20 ppm for a fumigation exposure period of 20 h to distinguish a susceptible *R. dominica* adult by death from a resistant beetle that survives the treatment. Results from the study showed that 32 out of the 34 geographic populations surveyed had beetles resistant to phosphine, and the frequency of resistance varied from 97% in a population from Parlier, California to 0% in beetles from both Carnduff, Saskatchewan and Starbuck, Manitoba. A 20-hour dose response assay was used to characterize the level of resistance by calculating the resistance ratio factors using beetles from a laboratory susceptible strain and those from five of the populations sampled. This resistance ratio (RR) was based on the ratio of $LC_{50}$ (estimate for the concentration to kill 50% of a test group) in the sampled population to the $LC_{50}$ for the susceptible strain. The highest RR for the five resistant populations was nearly 596-fold in beetles from Belle Glade, Florida, which represented the “strong” resistance phenotype, whereas the lowest RR in that group was 9-fold in Wamego, Kansas, representing the “weak” resistance phenotype. Manipulation of concentration and exposure periods can be utilized to manage strongly resistant *R. dominica* populations. The effect of several phosphine concentrations and fumigation exposure periods were assessed on progeny of

mixed life stage colonies of the strongly resistant *R. dominica*. A 48 hours dose response assay was carried out on these two strongly resistant populations to re-characterize their levels of resistance. Results from this assay showed that a phosphine dose of 730-870 ppm could control all resistant adult *R. dominica.* Additionally, phosphine concentrations ranging from 400-800 ppm phosphine for 96 hours completely killed mixed life stage colonies of strongly resistant lesser grain borers from the two populations studied. Lastly, fumigations done beyond 4 days at phosphine concentrations between 450-700 ppm controlled all phosphine resistant populations of lesser grain identified from our previous work. Phosphine applied at high concentrations is known to elicit a knockdown effect that can vary between susceptible and resistance grain insects. Using 18 of the 34 *R. dominica* populations this study sought to determine among three knockdown time (KT) techniques which method had potential to be utilized in an effective rapid assay for phosphine resistance in *R. dominica*. Adult *R. dominica* were exposed to a high concentration of phosphine (3000 ppm) to assess the time to knockdown 50%, 100% of a group of ten insects and that of single insects from the 18 geographically distinct populations vis a vis the resistance frequencies using the FAO method. $KT_{100}$ quick test was better than the $KT_{50}$ and $Kt_{single}$, because bioassays were able to clearly distinguish among susceptible, weak and strong resistant individuals. Time for $KT_{100}$ from susceptible populations did not exceed 30 minutes, while resistant populations had $KT_{100}$ times above 30 min, with strong-resistant populations times longer than 100 min.

# Resistance of *Rhyzopertha dominica* (Coleoptera: Bostrichidae) to phosphine fumigation; geographic variation, high dose treatments and rapid assay assessment

by

Edwin Afful

B.S., University of Ghana, 2007
M.S., University of Ghana, 2010

A DISSERTATION

submitted in partial fulfillment of the requirements for the degree

DOCTOR OF PHILOSOPHY

Department of Entomology
College of Agriculture

KANSAS STATE UNIVERSITY
Manhattan, Kansas

2018

Approved by:

Major Professor
Dr. Thomas W. Phillips

# Copyright

# Abstract

The emergence of heritable high-level resistance to the fumigant gas phosphine in stored-product insects is of grave concern to many grain growing countries around the world. The research reported in this dissertation was designed to determine the presence of phosphine resistance in 34 field collected populations of *Rhyzopertha dominica* (F.) from the United States and Canada, the potential to control resistant *R. dominica* populations utilizing high dose and longer exposure time strategies, and to develop a rapid assay for phosphine resistance detection. Using a discriminatory dose assay called the FAO number 16 method, adult *R. dominica* were sampled and subjected to a phosphine dose of 20 ppm for a fumigation exposure period of 20 h to distinguish a susceptible *R. dominica* adult by death from a resistant beetle that survives the treatment. Results from the study showed that 32 out of the 34 geographic populations surveyed had beetles resistant to phosphine, and the frequency of resistance varied from 97% in a population from Parlier, California to 0% in beetles from both Carnduff, Saskatchewan and Starbuck, Manitoba. A 20-hour dose response assay was used to characterize the level of resistance by calculating the resistance ratio factors using beetles from a laboratory susceptible strain and those from five of the populations sampled. This resistance ratio (RR) was based on the ratio of $LC_{50}$ (estimate for the concentration to kill 50% of a test group) in the sampled population to the $LC_{50}$ for the susceptible strain. The highest RR for the five resistant populations was nearly 596-fold in beetles from Belle Glade, Florida, which represented the “strong” resistance phenotype, whereas the lowest RR in that group was 9-fold in Wamego, Kansas, representing the “weak” resistance phenotype. Manipulation of concentration and exposure periods can be utilized to manage strongly resistant *R. dominica* populations. The effect of several phosphine concentrations and fumigation exposure periods were assessed on progeny of

mixed life stage colonies of the strongly resistant *R. dominica*. A 48 hours dose response assay was carried out on these two strongly resistant populations to re-characterize their levels of resistance. Results from this assay showed that a phosphine dose of 730-870 ppm could control all resistant adult *R. dominica.* Additionally, phosphine concentrations ranging from 400-800 ppm phosphine for 96 hours completely killed mixed life stage colonies of strongly resistant lesser grain borers from the two populations studied. Lastly, fumigations done beyond 4 days at phosphine concentrations between 450-700 ppm controlled all phosphine resistant populations of lesser grain identified from our previous work. Phosphine applied at high concentrations is known to elicit a knockdown effect that can vary between susceptible and resistance grain insects. Using 18 of the 34 *R. dominica* populations this study sought to determine among three knockdown time (KT) techniques which method had potential to be utilized in an effective rapid assay for phosphine resistance in *R. dominica*. Adult *R. dominica* were exposed to a high concentration of phosphine (3000 ppm) to assess the time to knockdown 50%, 100% of a group of ten insects and that of single insects from the 18 geographically distinct populations vis a vis the resistance frequencies using the FAO method. $KT_{100}$ quick test was better than the $KT_{50}$ and $Kt_{single}$, because bioassays were able to clearly distinguish among susceptible, weak and strong resistant individuals. Time for $KT_{100}$ from susceptible populations did not exceed 30 minutes, while resistant populations had $KT_{100}$ times above 30 min, with strong-resistant populations times longer than 100 min.

# Table of Contents

# List of Figures

# List of Tables

# Acknowledgements

I am very grateful to the source of life Jehovah God for providing me with strength, courage and endurance throughout my time at K-state. To Dr. Thomas Phillips my very caring, loving and fatherly advisor I cannot thank you enough for all you have done for me during my studies. You gave me the opportunity to make it over to the United States, guided me through it all and ensured I could got this work done. My sincere appreciation to my advisory committee members: Dr. James Campbell, Dr. Kun Yan Zhu, Dr. Manoj Nayak, and Dr. Bhadriraju Subramanyam for their time, encouragement, and support. To current and past members of Dr. Phillips's laboratory: Michael Aikins, Salehe Abbar, Barbara Amoah, Stephen Losey, Aaron Cato, Naomi Manu, Jacqueline Maille, Dr. Mukti Ghimire, Dr. Zhaorigetu Chen, and Dr. Ozgür Saglam I say thanks for your comradeship. Special thanks to Stephen and Aaron for all your time during our many field trips. Also sincere thanks to Tesfaye Tedaye and Erick Cordiero for their expertise in data analysis. To the many friends I made in Kansas I say thanks for your support. To my spiritual brothers and sisters of the Manhattan Congregation of Jehovah's witnesses I say thanks for everything and words cannot just describe how much you were there for me. I am sincerely grateful to the staff and faculty of the Department of Entomology. You all contributed to in many ways to the successful completion of my study, I would also like to thank my sources of funding, CRC Plant Biosecurity and the Kansas State Department of Entomology. Lastly, I would like to my wife, mom and sister for their continuous prayers and support throughout my studies.

# Dedication

I dedicate this work to my wife, Dorcas Afful, who sacrificed a lot during the last five years. I love you hon and it’s done now.

# Chapter 1 - Literature Review and Objectives

# Literature Review

## Stored grain insect pests

Agriculture plays an important role in the lives of the humans around the world as it contributes significantly to the economy of most countries. Produce from agriculture such as cereal grains and legumes when in storage are lost due to poor storage facilities, and attacks by insects, rodents and diseases. Insect pests are considered a major factor that reduce the quantity and quality of food (Gwinner et al. 1990). Depending on the type of grain, climatic factors and other ecological conditions it has been estimated that losses associated with insect pests could be estimated to be 15-50% in under developed countries (Pimentel 1978). In the United States, annual postharvest losses due to insects in corn and wheat were estimated at about $1.25 to $2.5 billion, accounting for 5 to 10% of the total value of corn and wheat produced (USDA, 2005). In the state of Indiana, the economic losses caused by insect damage in stored products were estimated at $12 million in 1990 (Mason et al. 1994).

The biology of stored product insects contribute primarily to such losses as these insect species can complete their life cycles in a short period of time (30-35 days) under optimum conditions producing several offspring that continue to damage stored grain. Losses associated with insects are not restricted to direct consumption of kernels, but include contamination such as accumulations of frass, exuviae, webbing, and insect cadavers. Additionally, insect activities cause changes in the storage environment which may cause warm, moist spots suitable for the development of microorganisms that result in further losses (Barak and Harein 1981, Subramanyam and Harein 1989).

Generally, stored products pests are categorized into two major groups based on their feeding habits: primary and secondary pests. Primary pests cause damage to stored grains by

directly feeding on the intact and stable grain. Insects such as the lesser grain borer, larger grain borer, maize weevil and rice weevil are well known primary pests of stored grain. Secondary pests however attack grains that have been previously damaged by either primary pests or by any mechanical means such as poor threshing, drying and handling. They also attack processed commodities such as flour and milled rice, as well as value-added products made from the milled products. The commonly known secondary pests are the red flour beetle, rusty grain beetle, cigarette beetle, Indian meal moth, among others.

**The Lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae)**

The lesser grain borer *Rhyzopertha dominica* is generally thought to have originated from the Indian subcontinent because many other species from the bostrichid family have had their origins there (Chittenden 1911; Schwardt 1933 Potter, 1935). It is now a cosmopolitan pest especially in warm countries. *R. dominica* is a member of the family Bostrichidae known as auger beetles or, powder post beetles. It is a thermophilic pest and not successful in its development at temperatures less than 23°C.

The lesser grain borer is a major pest of wheat and rice around the world (Flinn et al. 2004; Hagstrum and Subramanyam 2006). They are also found on a wide variety of foodstuffs including beans, dried chilies, turmeric, coriander, ginger, cassava chips, biscuits and wheat flour (Wright et al. 1990). *R. dominica* have been trapped several kilometers away from food storage or processing facilities using pheromone-baited traps (Fields et al. 1993; Edde et al 2006).

*R. dominica* is a holometabolous insect, or it undergoes complete metamorphosis. The lifecycle includes four stages: egg, larva, pupa, and adult. Adults are 2.3 – 3.0 mm long, dark red

brown/black in color with a cylindrical body. The elytra have regular rows of coarse punctures covered with curved setae. When viewed from above, the head is not visible and has its antennae end in a distinctive 3-segmented club-shape. Females of *R. dominica* lay between 200 and 500 eggs in their lifetime which are laid loosely on grains under ideal environmental conditions (Astuti et al. 2013). The eggs are ovoid in shape, 0.6 mm in length, 0.2 mm in diameter, white when first laid, and turn rose to brown before hatching (Edde, 2012, Potter, 1935). The larvae are C-shaped, white to cream colored, with biting mouthparts and three pairs of legs. There are on average four instars in *R. dominica* (Potter 1935, Thompson 1966). Pupa is about 3.9 mm long, white to brownish white in color and stage lasts about 8 days at 25ºC and 5–6 days at 28ºC. The fastest rate of development occurs at 34°C; at this temperature the egg takes 2 days, the larvae 17 days, and the pupae 3 days to complete development. Adults are long lived and can survive for 4-8 months.

*R. dominica* is a primary insect pest of stored grain with adults feeding on whole or cracked grain with larvae developing inside kernels. Adults lay eggs loosely near intact kernels and first instar larvae chew through the kernel and all larval stages and pupae complete development within kernels, reducing them to hollow husks. The early instar larvae are mobile outside of grain and within grain but become more immobile as they complete their development within the grain (Guedes et al. 1996). Adult males produce an aggregation pheromone in the frass that attracts both male and female adults (Williams et al. 1981). Adults are good flyers and can be trapped in pheromone-baited flight traps placed several kilometers from grain stores (Winterbottom 1922; Edde 2012).

**Management of *Rhyzopertha dominica***

Management of *R. dominica* involves a variety of pest management options including cultural practices that minimize pest build-up or migration into storage structures, such as cleaning of bins and equipment before harvest, sealing structures, cleaning up grain spills on the grounds, and spraying of bins and storage structures before storing grain. In addition to such techniques, the close monitoring of grain temperature and physical control measures such as grain drying and cooling below 15 °C to lower insect activity are important preventive measures.

Currently, the most effective tool used in controlling grain insect pests are chemical treatments, which include fumigants to mitigate current infestations. Direct application of contact insecticides to grains to provide protection from invading pests over a long period of time is also performed. These types of chemicals are referred to as grain protectants. Grain that is going to be stored for several months will often benefit from the use of a grain protectant. These materials are added to the grain as it is placed in storage. The use of grain protectants in the management of *R. dominica* is the most difficult relative to other insects in many countries (Collins, 2006, Lorini and Galley, 1999, Zettler and Cuperus, 1990). This is because of either resistance or are not effective against the insect. It has been reported that *R. dominica* has developed resistance to all approved organophosphorus insecticides – chlorpyrifos methyl, fenitrothion, pirimiphos methyl, and malathion (Collins 2006; Lorini and Galley 1999; Guedes et al., 1996, 1997, Navarro et al.; 1986; Zettler and Cuperus, 1990). Pyrethroids, which have proven to be successful against most insect pests, have been reported to be ineffective against *R. dominica* due to widespread resistance (Collins 2006, Lorini and Galley, 1996,1999).

The direct application of fumigants is a way of controlling *R. dominica* when infestations of *R. dominica* have been established and increased to an action threshold density within stored

grain. Fumigants are gaseous poisons that kill insects as the gas enters their bodies through the spiracles. Generally, fumigants are considered very effective in the management of stored grain insect pests. Effective fumigants are toxic to all life stages of insects, readily available and economical to use, leaves little or no harmful residue to commodities and are non-injurious to product quality, seed germination, and end-use quality. Phosphine, which has been the most common fumigant used for decades, and the more recently registered sulfuryl fluoride, are two examples of fumigants

**Phosphine**

Phosphine is a fumigant gas with such ideal properties that it has remained in use for over 60 years. Phosphine possesses the following attributes that make it ideal and widely used for grain fumigation:

- It does not leave toxic residues in treated communities
- Phosphine used properly poses no threat to the viability of seeds
- Gas is easily generated from metal phosphide products upon exposure to moist air at warm temperatures
- Phosphine is relatively inexpensive when compared to other fumigants, easy to apply in the solid formulations, and can be used on a wide range of storage types and commodities (Chaudhry 2000).

Phosphine is therefore used for disinfestation of stored seed- and food-grains, high-value commodities such as coffee beans and tobacco, dried fruits and certain processed foodstuffs. Additionally, phosphine is also used for the disinfestation of empty buildings and food processing facilities such as flour mills (Chaudry 2000).

Phosphine or hydrogen phosphide ($PH_3$) has a low molecular weight and boiling point so can diffuse rapidly and penetrate deeply into materials, such as large bulks of grain or tightly packed materials (Weast 1987). It is a colorless, flammable, extremely toxic gas, with a disagreeable, garlic-like odor. The gas is produced from formulations of metallic phosphides (usually aluminum or magnesium phosphide) that contain additional materials (inert in nature) for regulating release of the gas. Both metals exist as yellowish to dark grey and chartreuse crystals (World Health Organization [WHO] 1988). These solids are stable when dry but react with water as shown below to produce phosphine gas (Bond, 1984).

Aluminum Phosphide: $-AlP + 3H_20 \quad PH_3 + Al(OH)_3$

Mangesium Phosphide (faster gas release than AlP): $-Mg_3P_2 + 6H_20 \quad 2PH_3 + 3Mg(OH)_2$

Aluminum and magnesium phosphide are formulated as tablets, pellets or small sachets and these formulations generate gas when in contact with moist air or moisture from grain and other commodities at proper temperatures (Bond 1984). Sealing of the space that is to be fumigated is key for effective use of phosphine. The mode of action of this potent toxin against many stored-product insects is currently not known. Studies have proposed that phosphine has three modes of action: disruption of the sympathetic nervous system, suppressed energy metabolism, and toxic changes to the redox state of the cell (Nath et al. 2011). It has also been suggested that phosphine toxicity is reliant on molecular oxygen (Bond and Monro, 1967; Kashi, 1981; Cheng et al., 2003). Other studies have assumed that phosphine interrupts with normal oxygen metabolism in insects, causing production of highly injurious ‘oxyradicals’ (Bolter and Chefurka 1990; Chaudhry and Price 1992). These radicals cause disorder to vital proteins and enzymes, resulting in mortality of the target insects (Chaudhry and Price 1992). Phosphine gas has been reported to be corrosive to many metals including silver, gold, and most importantly

copper, especially at high humidity (Bond et al. 1984). Due to its corrosiveness to copper, phosphine is not normally used in facilities such as mills and similar food processing facilities which could have equipment and other electrical fixtures with copper that could be damaged at great loss.

As with many fumigants, successful phosphine fumigations are affected by length of exposure time, concentration of phosphine and temperature. Phosphine gas is slow acting and generally more effective at low concentrations, with exposure times of four or more days required for effective fumigations for most species (depending on temperature) (Bond 1984). To maintain doses that are lethal to the stored grain insects it is very important to have an air-tight storage structure during fumigation (Chaudhry 1997). The inability to maintain the lethal dose due to leaks in the structure, will result in insect populations not fully controlled. Such sub lethal exposures if repeated frequently, will result in the selection of phosphine resistant individuals (Chaudhry 1997).

**Phosphine Resistance**

Repeated fumigations with phosphine in leaky structures has resulted in under-dosing and exposing stored grain insects to sub-lethal doses of the toxin. The survival of insects with phosphine-resistant genes will enable them to continue breeding, passing on their resistance genes to subsequent generations. Such repeated fumigations may kill susceptible insects but will favor the insects that carry the resistance genes (Flinn et al 2003).

During 1972-1973 a global survey was undertaken by the Food and Agriculture Organization (FAO) to assess the presence or absence of stored grain insects resistant to phosphine. The results of the survey showed that about 10 % of the collected populations

contained phosphine-resistant individuals (Champ and Dyte 1976). Since that time however, widespread resistance to phosphine has been reported in several species of stored-product insects in many countries in which phosphine control failure has been detected. Countries such as Morocco (Benhalima et al. 2004), Brazil (Ansell et al.1990; Athie and Mills 2005), Vietnam (Bui 1998); China (Cao 2006); Australia (Daglish, 1998) and Pakistan (Alam et al. 1999) have reported high levels of phosphine resistance in different insect species. Phosphine resistance is now known to be present in at least 11 species of stored-product insects in 45 countries, and the list is growing.

It has been suggested that one mechanism of resistance is the ability to lower respiration in resistant individuals (Benhalima et al. 2004), and it was once presumed that a detoxification process within the insects existed (Chaudhry 1997). Two resistance phenotypes have been identified: weak and strong. Detailed genetic studies in Australia have identified two loci responsible for phosphine resistance, referred to as rph1 and rph2. The rph1 locus, first described in the red flour beetle *Tribolium castaneum* (Herbst), is responsible for weak resistance, whereas the second, rph2, acts synergistically with rph1 to confer strong resistance (Schlipalius et al. 2002). Schlipalius et al. (2012) discovered that mutations in the gene coding for dihydrolipoamide dehydrogenase (DLD) in *R. dominica* (F.) and *T. castaneum* are the cause of phosphine resistance at the rph2 locus in both species.

Resistance to phosphine in *R. dominica* is an ongoing problem. In Australia, strongly resistant *R. dominica* was first detected in southern Queensland in 1999 (Collins 1998). Studies in Brazil detected resistance in the lesser grain borer in the early 1990s (Taylor 1989; Ansell et al. 1990; Pacheco et al. 1990; Sartori et al. 1990). In Morocco, Benhalima et al. (2004) examined the levels of resistance in 51 populations of three different insect species due to reports of failed

fumigations. Most of these populations, especially the populations of *R. dominica* and *T. castaneum*, showed survival from 50-80%.

In North America, limited research was carried out on resistance to phosphine after the Champ and Dyte (1976) survey. Zettler et al. (1989) reported variable resistance levels in each of three different species of pests surveyed in peanut storage facilities in the US states of Alabama, Georgia, and Florida. In Oklahoma, phosphine resistant individuals were found in several populations of *T. castaneum* and *R. dominica* infesting stored wheat a year after the study in those three states (Zettler and Cuperus 1990). *R. dominica* populations showed a higher prevalence of resistance with 8 of 21 populations showing resistance. A study was carried out recently by Opit et al. (2012) to determine if there had been changes to resistance frequencies from the same sites in Oklahoma that were studied 20 years prior. Results showed that all five populations of *R. dominica* assessed were resistant to phosphine indicating a increase in frequencies relative to what was measured 20 years earlier. A dose–response assay on the *R. dominica* populations were 1500-fold compared with a susceptible strain. In Canada the situation is different as there are little published data on phosphine resistance since Champ and Dyte (1976)

**Methods of Detecting Phosphine Resistance**

Detection of phosphine resistant individuals in many stored product insect population relies on the method developed in 1970 and used by Champ and Dyte (1976), called FAO method number 16 (Food and Agriculture Organization 1975). Adult of resistant and susceptible insects were distinguished from each other based on discriminating doses for each of the seven different pests of stored grain tested during the survey. These doses were based on the $LD_{99.9}$ for

the susceptible strains using probit regression. The method stipulated that adult insects were to be exposed to a discriminating dose of phosphine for 20 hours at 25°C and then left in fresh air for 14 days to allow for recovery or delayed mortality. Insects that survived this 20-hour exposure and the 14-day post fumigation were considered resistant, while dead insects were scored as susceptible. This method, although efficient at reporting the presence or absence of resistant individuals in any given population, has many disadvantages. One limitation is the wait time of 14 days needed to ascertain the level of resistance in a given insect population. Other disadvantages include how laborious and technical this method is. These demerits of the FAO method have resulted in interests in developing simpler and more rapid assays for phosphine resistance detection.

The behavior of adults on exposure to phosphine has been explored in the development of rapid assays for the detection of phosphine resistance. Bang and Telford (1966), in assessing the effect of sub-lethal doses of fumigants, reported that when insects are exposed to certain concentrations of phosphine for a short time they would enter a state of narcosis in which they stopped moving or get "knocked down", but would then recover and resume activity in the absence of the gas. The study also indicated that as the concentration of phosphine was increased, the time to narcosis decreased. In a similar study by Winks (1985), the narcotic effect of phosphine concentrations ranging 0.2 to 50 mg/L on adult *T. castaneum* were assessed. The results from this study corroborated that of Bang and Telford that the time to narcosis decreased at higher concentrations. Results of both studies gave a key factor utilized in the development of rapid assays which is knockdown. This behavior of stored grain insects to various concentrations of phosphine led to another factor utilized in rapid assay assessment which is the time it takes for these insect species to enter the state of narcosis.

Using adult *T. castaneum*, Winks (1985) assessed the significance of response time in the detection of measurements of phosphine toxicity. Further questions were asked to know if there were any difference between the time to knockdown of susceptible and resistant insect populations using the FAO method. Reichmuth (1991) answered this question by comparing the time to knockdown between resistant and susceptible *Rhyzopertha dominica*. Results from his study showed that 13 resistant *R. dominica* took longer to knockdown than susceptible beetles. Waterford and Winks (1994), followed this study by comparing the knockdown time of individual *T. castaneum* that were either resistant or susceptible to phosphine. Additionally, they crossed individuals expressing these phenotypes based on their varying the time-to knockdown. Results from their study showed that the crosses between resistant individuals had a higher knockdown time compared to crosses of the most susceptible individuals after twenty generations.

Reichmuth (1991) devised a quick test based on the behavior of *R. dominica* and time to knockdown to different concentrations of phosphine. In his test, *R. dominica* with known status of resistance were exposed to a concentration of phosphine ranging from 1000-3000 ppm and then checked after 30 minutes. Insects that remained active after that period were categorized as resistant (Reichmuth 1991). Bell et al. (1994) designed a same day test to identify resistance for three species of stored product beetles, based on their mobility on paper cones during exposure to phosphine. Using similar methods as Reichmuth (1991) the concentration employed in this test were much lower (221-299 ppm). The ability of adult of these three species to retain their grip on the sloping side of the cone, fall to the bottom and were unable to stand were considered to be knockdown. Bell et al. (1994), despite using lower concentrations, were able to discriminate between resistant and susceptible species using their methods. Cao and Wang (2000) examined the relationship between $LC_{50}$ of three adult insect species [*T. castaneum*, rice weevil *Sitophilus*

*oryzae* (L.) and maize weevil *Sitophilus zeamais* (Motsch.)] exposed to phosphine (PH3) according to the FAO method and time to 50% knockdown ($KT_{50}$) at 1438 ppm. In this experiment knockdown was defined as when the adults were not able to climb or walk properly. Nayak et al. (2013) developed a time-to-knockdown bioassay method using a concentration of 1400 ppm, using rusty grain beetle *Cryptolestes ferrugineus* (Stephens). At designated time intervals, the insects were tipped onto a white filter paper for visual assessment of knockdown. The criterion of response was the inability of the insect to walk in a coordinated manner. Based on this they found that the time intervals to 99.9% knockdown were 14.65 min for the phosphine susceptible, 309.6 min for the weakly resistant and 22,783 min for the strongly resistant. Steuerwald et al. (2006) used a phosphine concentration of 3000 ppm and a fixed amount of exposure time to judge the susceptibility of 5 different insect species. Status of resistance is scored based on whether beetles are "active" within the syringe containing phosphine.

Molecular and genetic methods have played a key role in the development of assays for the detection of resistance. The identity of the mutation in the gene conferring resistance to phosphine sparked research into such molecular assays. Schlipalius et al. (2012) identified a point mutation in a core metabolic enzyme, dihydrolipoamide dehydrogenase (DLD) which confers strong resistance in these species. Chen et al. (2015) used two restriction enzymes MboI and BstNI, to be able to differentiate presence or absence of the resistant (R) allele from PCR products based on this single mutation. This allowed for inference of resistamce genotypes with that allele.

**Management of Phosphine Resistance**

The increasing levels of strong phosphine resistant individuals in many species of stored grain insects have called into question the long-term viability of phosphine as fumigant for pest management. As with most pesticides, the development of resistance will require a change to a new active ingredient. Several studies have investigated the potential of other fumigant gases such as ozone, sulfuryl fluoride, propylene oxide, ethyl formate, chlorine dioxide among others as an alternative to phosphine (Xinyi et al 2017; Opit et al 2016; Isikber et al. 2006; Haritos et al. 2006). These fumigants have not achieved as much success as phosphine in the field.

To protect the continued use of phosphine in the grain storage industry and continue to market low residue product in the world grain trade, it is important to manage development of phosphine resistance in stored-grain insects (Newman 1998). Phosphine concentration, exposure period and grain temperature are major variables that determine the toxicity of phosphine. These variables can be manipulated to increase the toxicity to phosphine (Bell, 1976; Lindgren and Vincent 1966; Hole et. al 1976; Bond 1984). Additionally, several studies have shown that the manipulation of concentration and exposure periods can be utilized to manage strong resistant populations (Daglish et al 2002; Ho and Wink 1995; Winks 1984; Manivannan 2015). A study by Kaur and Nayak (2015) to develop robust fumigation protocols that could be used in a range of practical grain storage situations to control strongly resistant *C. ferrugineus* populations by manipulating concentrations and temperature was carried out in Australia. The study established fumigation protocols in the laboratory for three phosphine concentrations (1.0, 1.5 and 2.0 mg/L) and three temperatures (25, 30 and 35 °C) for the control of all life stages of strongly resistant *C. ferrugineus*. Findings of the study stipulated that a successful control of resistant *C.*

*ferrugineus* can be achieved using shorter fumigation periods at elevated grain temperatures, irrespective of the concentration used.

**Objectives**

The general objective of this study was to determine presence of phosphine resistance in 34 field collected populations of *R. dominica* from the United States and Canada, study the potential for managing resistant *R. dominica* populations utilizing high dose and longer exposure days strategies, and to develop a rapid assay for phosphine resistance detection. Specific objectives were as follows:

1. Determine the frequency of phosphine resistance in geographically separated populations of *R. dominica* across the United States and Canada using a standard discriminating dose bioassay.
2. Ascertain the presence of weak- or strong-resistant beetles within some populations and to characterize these populations using a comparative dose–response assay.
3. Characterize strong resistance in previously studied *R. dominica* populations with dose mortality experiments of adults exposed for 48 h.
4. Evaluate efficacy of both high and low concentrations of phosphine applied to mixed life stage samples for 48 h to representative weak resistant and strong resistant populations.
5. Determine the number of days of exposure needed to control representative strong resistance populations exposed to either high or low concentrations of phosphine.
6. Assess the concentrations of phosphine needed to elicit the quickest knock down times among adult *R. dominica* populations classified as susceptible, weakly and strongly resistant.

7. Determine the effect of applying a physical stimulus on knockdown times for individual beetles held under a high phosphine concentration.
8. Determine an efficient knockdown time technique using single beetles or groups of adults from *R. dominica* populations that will give information comparable to the well-known FAO method, but in less than a day's time.
9. Assess the relationship between knockdown time and recovery time at a given phosphine concentration for *R. dominica* populations classified as susceptible, weakly and strongly resistant.

## References

**Alam, M. S., Ahmed, M., Iqbal, S. and A. Ahmad. 1999.** A survey of resistance to phosphine in some coleopterous pests of stored wheat and rice grain in Pakistan. Pakistan Journal of Biological Sciences (Pakistan).

**Ansell M. R., C.E. Dyte and R.H. Smith**. **1990** The inheritance of phosphine resistance in *Rhyzopertha dominica* and *Tribolium castaneum* F. Fleurat Lessard, P. Ducom (Eds.), Proceedings of the Fifth International Working Conference on Stored-product Protection. Bordeaux, France, 9–14 September 1990, Institut National de la Recherche Agronimique, Paris, France. pp. 961-970

**Astuti, L., G. Mudjiono., S. Rasminah, and B. Rahardjo. 2013.** Influence of temperature and humidity on the population growth of *Rhyzopertha dominica*(F.) (Coleoptera: Bostrichidae) on milled rice. Journal of Entomology10,86–94.

**Attia, F. I. and H. G. Greening. 1981.** Survey of resistance to phosphine in coleopterous pests of grain and stored products in New South Wales. Gen. Appl. Entomol. 13: 93-97.

**Attia, F. I. 1983.** Insecticide and fumigant resistance in insects of grain and stored-products in Australia, pp. 196-208. In Proc. 3rd Int. Nork. Conf. on stored Prod. Ent. Manhattan, Kansas, Oct. 1983.

**Bang Y. H. and H. S. Telford. 1966.** Effect of sub lethal doses of fumigants on stored-grain insects. Ph.D. Dissertation, University of Washington Agricultural Experiment Station. Tech. Bull. 50.

**Barak, A. V., and P. K. Harein. 1981.** Losses associated with insect infestation of farm-stored shelled corn and wheat in Minnesota. Univ. Minn. Agr. Exp. Sta. Misc. Publ. 12, 94p.

**Benhalima, H., M. Q. Chaudhry, K. A. Mills, and N. R. Price. 2004.** Phosphine resistance in stored-product insects collected from various grain storage facilities in Morocco. J Stored Prod Res 40: 241–249.

**Bell C.H. 1976**. The tolerance of developmental stages of four stored product moths to phosphine. J Stored Prod Res **12**: 77–86 (1976).

**Bell C.H. 1992.** Time concentration and temperature relationship for phosphine activity in tests on diapausing larvae of *Ephestia elutella* (Hübner) (Lepidoptera: Pyralidae). Pestic Sci 35: 255–264

**Bolter C.J., and W. Chefurka 1990.** Extramitochondrial release of hydrogen peroxide from insect and mouse liver mitochondria using the respiratory inhibitors phosphine, myxothiazol, and antimycin and spectral analysis of inhibited cytochromes. Archives of Biochemistry and Biophysics. 278(1):65–72.

**Bond E.J., Monro H.A.U. and Buckland C.T. 1967.** The influence of oxygen on the toxicity of fumigants to *Sitophilus granarius* (L) J Stored Prod Res. 3(4):289–294.

**Bond, E. J. 1984.** Manual of fumigation for insect control. FAO Plant Production and Protection Paper No. 54, FAO, Rome

**Bond, E. J., T. Dumas, and S. Hobbs. 1984.** Corrosion of metals by the fumigant phosphine. J Stored Prod. Res. 20: 57-63.

**Bui, C. H. 1998**. Some initial results on phosphine resistance of major product insect pests in Vietnam. In Stored Products Protection. Proceedings of the Seventh International Working Conference on stored product protection (pp. 14-19).

**Cao, Y. 2006.** Survey of the resistance of stored-grain pests to phosphine in China. J. Henan Univ. Technol. (Natural sci. edition) (in Chinese with English abst.), *27*(1), 1-6.

**Cao, Y. and D. Wang. 2000.** Relationship between phosphine resistance and narcotic knockdown in Tribolium castaneum (Herbst), *Sitophilus oryzae* (L.), and *S. zeamais* (Motsch), pp. 609-616. In Int. Conf. on Controlled Atmosphere and Fumigation in Stored Products, 29 October – 3 Novemeber, Fresno, CA. Printco Ltd., Nicosia, Cyprus.

**Champ, B. R. and C. E. Dyte. 1976.** Report of the FAO global survey of pesticide susceptibility of stored grain pests. FAO Plant Production and Protection Series 5. FAO, Rome

**Chaudhry M.Q. and N. Price 1992.** Comparison of the oxidant damage induced by phosphine and the uptake and tracheal exchange of 32P-radiolabelled phosphine in the susceptible and resistant strains of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) Pesticide Biochemistry and Physiology. 42(2):167–179.

**Chaudhry, M. Q. 1997.** A review of the mechanisms involved in the action of phosphine as an insecticide and phosphine resistance in stored-product insects. Pestic. Sci. 49: 213–228.

**Chaudhry M. Q. 2000.** Phosphine resistance. Pesticide Outlook 11(3), 88-91.

**Chaudhry M.Q., Bell H.A., Savvidou N., and MacNicoll A.D. 2004.** Effect of low temperatures on the rate of respiration and uptake of phosphine in different life stages of the cigarette beetle *Lasioderma serricorne* (F.). J Stored Prod Res 40: 125–134.

**Chen. Z., D. Schlipalius, G. Opit, B. Subramanyam, and T. W. Phillips. 2015.** Diagnostic molecular markers for phosphine resistance in U.S. populations of *Tribolium castaneum* and *Rhyzopertha dominica*. PLoS ONE 10(3): e0121343. doi:10.1371/journal.pone.0121343.

**Cheng Q, Valmas N, Reilly P.E.B., Collins P.J., Kopittke R., and P.R. Ebert. 2003.** *Caenorhabditis elegans* mutants resistant to phosphine toxicity show increased longevity and cross-resistance to the synergistic action of oxygen. Toxicological Sciences. 73(1):60–65.

**Chittenden F.H. 1911.**The lesser grain borer and the larger grain borer Bulletin of United State Bureau of Entomology, 96 (1911), pp. 29-47

**Collins, P. J., G. J. Daglish, M. Bengston, T. M. Lambkin, and H. Pavic. 2002.** Genetics of resistance to phosphine in *Rhyzopertha dominica* (Coleoptera: Bostrichidae). J. Econ. Entomol. 95: 862- 869.

**Collins, P.J. 2006.** Resistance to chemical treatments in insect pests of stored grain and its management I. Lorini, B. Bacaltchuk, H. Beckel, D. Deckers, E. Sundfeld, J.P. dos Santos, J.D. Biagi, J.C. Celaro, L.R.D.'A. Faroni, L.de O.F. Bortolini, M.R. Sartori, M.C. Elias, R.N.C. Guedes, R.G. da Fonseca, V.M. Scussel (Eds.), Proceedings of the 9th International Working Conference on Stored Product Protection, 15–18 October 2006, Sao Paulo, Brazil. pp. 277-282

**Daglish, G. J., and P. J. Collins 1998.** "Improving the relevance of assays for phosphine resistance." In Proceedings of the 7th International Working Conference on Stored Product Protection. Sichuan Publishing House of Science and Technology: Beijing, China, pp. 584-593.

**Daglish, G. J., Collins, P. J., Pavic, H. and R. A. Kopittke. 2002.** Effects of time and concentration on mortality of phosphine-resistant *Sitophilus oryzae* (L) fumigated with phosphine. Pest Manag. Sci.1015-1021.

**Edde P. A, T. W. Phillips, C. Nansen and M. E. Payton. 2006.** Flight Activity of the Lesser Grain Borer, *Rhyzopertha dominica* F. (Coleoptera: Bostrichidae), in Relation to Weather, Environ. Entomol. 616–624.

**Guedes, R.N.C., B.A. Dover and S. Kambhampati. 1996.** Resistance to chlorpyrifos-methyl, pirimiphos-methyl, and malathion in Brazilian and U.S. populations of *Rhyzopertha dominica* (Coleoptera: Bostrichidae) J. Econ. Entomol, 89, pp. 27-32

**Hole B.D., Bell C.H., Mills K.A. and G. Goodship. 1976.** The toxicity of phosphine to all developmental stages of thirteen species of stored product beetles.J Stored Prod ResRes 12: 235–244.

**Guedes, R. N. C., Kambhampati, S., Dover, B. A., and Zhu, K. Y. 1997.** Biochemical mechanisms of organophosphate resistance in Rhyzopertha dominica (Coleoptera: Bostrichidae) populations from the United States and Brazil. Bulletin of Entomological Research, 87(6), 581-586.

**Fields, P. G., Van Loon, J., Dolinski, M. G., Harris, J. L., and Burkholder, W. E. 1993.** The distribution of *Rhyzopertha dominica* (F.) in western Canada. Can. Entomol., 125(2), 317-328.

**Flinn, P. W., Hagstrum, D. W., Reed, C., and T. W. Phillips. 2004.** Simulation model of *Rhyzopertha dominica* population dynamics in concrete grain bins.J Stored Prod ResRes. 40(1), 39-45.

**Gwinner, J., Harnisch, R., and Műck, O 1990.** Manual on the prevention of post-harvest grain losses, post-harvest project, Pickhuben 4, 2000 Hambung,.

**Hagstrum, D. W. and Bh. Subramanyam. 2006.** Fundamentals of Stored Product Entomology, AACC International, St. Paul, MN

**Haritos, V. S., Damcevski, K. A. and G. Dojchinov 2006.** Improved efficacy of ethyl formate against stored grain insects by combination with carbon dioxide in a ‘dynamic’application. Pest Manag. Sci. 325-333.

**Isikber, A. A., Navarro, S., Finkelman, S. and M. Rindner.2006.** Propylene oxide: A potential quarantine and pre-shipment fumigant for disinfestation of nuts. Phytoparasitica, 34(4), 412-419.

**Kaur, R., and Nayak, M. K. 2015.** Developing effective fumigation protocols to manage strongly phosphine-resistant *Cryptolestes ferrugineus* (Stephens)(Coleoptera: Laemophloeidae). Pest Manag. Sci. 1297-1302.

**Kashi K. 1981.** Response of five species of stored-product insects to phosphine in oxygen-deficient atmospheres. J. Pest Sci. 111–115.

**Lindgren DL and L.E. Vincent 1966**. Relative toxicity of hydrogen phosphide to various stored-product insects. J. Stored Prod. Res 2: 141–146 (1966).

**Lorini I and D.J. Galley 1996.** Changes in resistance status of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), a pest of stored grain in Brazil, with and without deltamethrin selection Resistant Pest Management Newsletter. pp. 12-14

**Lorini I and D.J. Galley** 1999 Deltamethrin resistance in *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), a pest of stored grain in Brazil J Stored Prod Res, 35, pp. 37-45

**Mason, L. J., D. E. Maier, W. H. Adams, and J. L. Obermeyer.1994.** Pest management of stored maize using chilled aeration a mid-west united states perspective, pp. 312-317.In E. Highley, E. J. Wright, H. J. Banks, and B. R. Champ [eds.], Stored Products Protection: Proceedings of the 6th International Working Conference on Stored-Product Protection, 17-23 April 1994, Canberra, Australia. CAB International, Wallingford, United Kingdom.

**Navarro, S., Carmi, Y., Kashanchi, Y. and E. Shaaya. 1986.** Malathion resistance of stored-product insects in Israel. Phytoparasitica, 14(4), 273-280.

**Nayak, M. K. 2012.** Managing resistance to phosphine in storage pests: Challenges and opportunities, pp. 609-619. *In* Proc 9th. Int. Conf. on Controlled Atmosphere and Fumigation in Stored Products, 15-19 October, Antalia, Turkey. ARBER Professional Congress Services, Turkey.

**Nayak, M. K., Holloway, J. C., Emery, R. N., Pavic, H., Bartlet, J. and P.J. Collins.2013.** Strong resistance to phosphine in the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae): its characterisation, a rapid assay for diagnosis and its distribution in Australia. Pest Manag. Sci. 48-53.

**Nath, N. S., I. Bhattacharya, A. G. Tuh, D. I. Schlipalius, and P. R. Ebert. 2011.** Mechanisms of phosphine toxicity. J. Toxicol. 2011: 9 pages. Article ID 494168. http://dx.doi.org/10.1155/2011/494168.

**Newman, C. R. 2010.** A novel approach to limit the development of phosphine resistance in Western Australia. *Julius-Kühn-Archiv*, (425), 1038.

**Opit, J. P., T. W. Phillips, M. J. Aikins, and M. M. Hasan. 2012.** Phosphine resistance in *Tribolium castaneum* and *Rhyzopertha dominica* from stored wheat in Oklahoma. J. Econ. Entomol. 105: 1107-1114. 24

**Opit, G. P., Thoms, E., Phillips, T. W., and M. E. Payton. (2016).** Effectiveness of sulfuryl fluoride fumigation for the control of phosphine-resistant grain insects infesting stored wheat. J. Econ. Entomol. 109(2), 930-941.

**Pacheco, I. A., M. R. Santori, and R. W. D. Taylor. 1990.** Survey of phosphine resistance in stored grain insect pests in the state of Sao Paulo. Coletânea do Instituto de Tecnologia de Alimentos 20: 144-154.

**Pimentel, D. (1978).** Socioeconomic and legal aspects of pest control. Pest Control Strategies pp. 55-71.

**Potter C 1935**. The biology and distribution of *Rhyzopertha dominica* (Fab.) Transactions of the Royal Entomological Society of London, 83 pp. 449-482

**Reichmuth, C. 1991.** A quick test to determine phosphine resistance in stored-products research. GASGA Newsletter 15: 14-15.

**Reichmuth, C. 1994.** Uptake of phosphine by stored-product pest insects during fumigation, pp. 157-162, *In* Proceedings of the 6th International Working Conference on Stored-product Protection, 17-23 April 1994, Canberra, Australia. CAB International, Wallingford, United Kingdom

**Sartori, M. R., Pacheco, I. A. and R.M. Vilar. 1990.** Resistance to phosphine in stored grain insects in Brazil. In *International Working Conference on Stored-Product Protection* (Vol. 5, pp. 1041-1050).

**Sayaboc, P. D., A.J.G. Gibe, and F. M. Caliboso. 1998.** Resistance of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) to phosphine in the Philippines. Philipp. Entomol. 12: 91-95.

**Schlipalius D. I, Q. Cheng, P.E.B. Reilly, P.J. Collins, P.R. Ebert. 2002.**Genetic linkage analysis of the lesser grain borer *Rhyzopertha dominica* identifies two loci that confer high-level resistance to the fumigant phosphine Genetics, 161 (2002), pp. 773-782

**Schlipalius, D. I, N. Valmas, A. G. Tuck, R. Jagadeesan, L. Ma, R. Kaur, A. Goldinger, C. Anderson, J. Kuang, S. Zuryn, Y. S. Mau, Q. Cheng, P. J. Collins, M. K. Nayak, H. J. Schirra, M. A. Hilliard, and P. R. Ebert. 2012.** A core metabolic enzyme mediates resistance to phosphine gas. Science 338(6108): 807–810. 25

**Schwardt H.H. 1933.** Life history of the lesser grain borer Journal of the Kansas Entomological Society, 2 pp. 61-66

**Subramanyam, B., and P. K. Harein. 1989**. Insects infesting barley stored on farms in Minnesota J. Econ. Entomol. 82:1817-1824.

**Steuerwald, R., H. Dierks-Lange, and S. Schmitt. 2006.** Rapid bioassay for determining the phosphine tolerance, pp. 306-311. In Proceedings of the 9th International Working Conference on Stored-Product Protection, 15 - 18 October 2006, Campinas, São Paulo, Brazil. Brazilian Post-harvest Association - ABRAPOS, Passo Fundo, RS, Brazil.

**Thompson, V.** Biology of the lesser grain borer, *Rhyzopertha dominica* (F) Bulletin of Grain Technology, 4 (1966), pp. 163-168

**Waterford, C. J., and R. G. Winks. 1994.** Correlation between phosphine resistance and narcotic response in *Tribolium castaneum* (Herbst), pp. 221-225. *In* Proceedings of the 6th International Working Conference on Stored-product Protection, 17-23 April 1994, Canberra, Australia. CAB International, Wallingford, United Kingdom.

**Weast, R. C. 1987.** Handbook of Chemistry and Physics, 67th edn. CRC Press Inc., Boca Raton FL.

**Williams H.J. Silverstein R.M. Burkholder W.E. and A. Khorramshahi. 1981.** Dominicalure 1 and 2: components of aggregation pheromone from male lesser grain borer *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae). J. Chem. Ecol. 7: 759–781.

**Winterbottom, D.C. 1922**. Weevil in Wheat and Storage of Grain in Bags. A Record of Australian Experience during the War Period (1915 to 1919) Government Printer, North Terrace, Adelaide, Australia.

**Xinyi, E., Subramanyam, B., and B. Li, 2017.** Responses of phosphine susceptible and resistant strains of five stored-product insect species to chlorine dioxide.J Stored Prod ResRes 72, 21-27.

**Zettler, J. L., W. R. Halliday, and F. H. Arthur. 1989.** Phosphine resistance in insects infesting stored peanuts in the southeastern United States J. Econ. Entomol. 82: 1508-1511.

**Zettler, J. L. and G. W. Cuperus. 1990.** Pesticide resistance in *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae) in wheat. J. Econ. Entomol. 83: 1677-1681.

# Chapter 2 - Phosphine Resistance in North American Field Populations of the Lesser Grain Borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae)

## Abstract

Phosphine is the most widely used fumigant for stored grain insect pests, and resistance to phosphine has evolved in several species worldwide. This study was designed to determine the presence of phosphine resistance in 34 populations of *Rhyzopertha dominica* (F.) collected from the United States and Canada. Adult *R. dominica* were sampled and subjected to a discriminatory dose toxicity assay consisting of exposure to 20 ppm of phosphine for 20 h to distinguish a susceptible *R. dominica* adult by death from a resistant beetle that survives the treatment. All but two of the 34 geographic populations surveyed had some beetles that were resistant to phosphine, and the frequency of resistance varied from 97% in a population from Parlier, California to 0% in beetles from both Carnduff, Saskatchewan and Starbuck, Manitoba. Probit analyses of dose-mortality bioassays with beetles from a laboratory-susceptible strain and those from five of the populations sampled were used to calculate resistance ratio factors (RRs) based on the ratio of $LC_{50}$ (estimate for the concentration to kill 50% of a test group) in the sampled population to the $LC_{50}$ for the susceptible strain. The highest RR for the five resistant populations was nearly 596-fold in beetles from Belle Glade, Florida, whereas the lowest RR in that group was 9-fold in Wamego, Kansas. This study revealed that phosphine resistance in *R. dominica* is common across North America and some populations have levels of resistance that may pose challenges for continued use of phosphine for their management.

# Introduction

Fumigation has been used routinely over the past several decades as a primary component in the management of insects and other pests in stored grains. Hydrogen phosphide, commonly known as phosphine, with the formula $PH_3$, is a fumigant gas with properties that have made it a major stored grain insecticide (Chaudhry 2000). Phosphine is relatively inexpensive, easy to apply, and can be used for a wide range of storage structures and commodities (Chaudhry 2000, Kaur and Nayak 2015). In addition to these attributes, phosphine has been accepted globally as a relatively residue-free and food-safe treatment for grain (Nayak et al. 2015). Over-reliance on this fumigant by the grain, food, and pest control industries has likely contributed to the development of genetically based resistance to phosphine in major species of stored product insects.

The first global survey of phosphine resistance was conducted during the 1970s by Champ and Dyte (1976) who used a discriminating dose bioassay on adults (FAO 1975) and documented the occurrence of phosphine resistance in several key stored grain pest species across many countries. More recent work has confirmed high frequencies of phosphine resistance in key pest species in numerous locations in Australia (Nayak et al. 2013), India (Kaur et al. 2015), Brazil (Lorini et al. 2007), and the United States (Opit et al. 2012, Chen et al. 2015). These recent studies have gone beyond simply documenting the presence or absence of phosphine resistance in a pest population. They have revealed additional information on the occurrence of two general phenotypes for either ‘strong’ phosphine resistance in individuals that can tolerate extremely high concentrations of phosphine and also a ‘weak resistance’ phenotype for insects that could be killed at phosphine concentrations just a few fold greater than concentrations needed to kill susceptible insects (Lorini et al. 2007, Opit et al. 2012, Nayak et al.

2013). Significant progress has also been made in bioassay methods that can classify phosphine resistance as either weak or strong phenotypes in some species (Nayak et al. 2013). The genetic locus responsible for strong phosphine resistance, a phenotype that requires resistance alleles for weak resistance to be fixed at another locus, was recently reported (Schlipalius et al. 2012) and that information then allowed for having molecular diagnostics to enable researchers to determine the presence of strong-resistant genes occurring in populations with resistant insects (Chen et al. 2015).

Studies on phosphine resistance in North American stored grain insect pests have been reported to various extents over the past 40 years. Champ and Dyte (1976) detected low frequencies of phosphine resistance in three out of five beetle species surveyed in North America. Zettler et al. (1989) reported variable resistance levels in each of three different species of pests surveyed in peanut storage facilities in the US states of Alabama, Georgia, and Florida. A study in Oklahoma at the same time found phosphine resistance in several populations each of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), the red flour beetle and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae), the lesser grain borer, infesting stored wheat (Zettler and Cuperus 1990). Opit et al. (2012) collected *T. castaneum* and *R. dominica* from some of the same sites in Oklahoma that had been studied 20 years prior by Zettler and Cuperus (1990) to determine whether there had been any changes in resistance frequency over that time period. That study found that resistance frequencies at some Oklahoma locations were significantly higher than those measured 20 years earlier. A dose–response assay on several populations indicated the levels of resistance of several hundred-fold compared with a susceptible strain. Opit et al. (2012), therefore, recommended that future research should determine the extent of different pest species and geographic locations that manifest phosphine

resistance in North America. Towards that end, a recent study by Cato et al. (2017) reported nearly half of the 25 *T. castaneum* populations sampled had some resistance as measured using a discriminating dose bioassay (FAO 1975), and they occurred in such distantly separated locations as Florida, Texas, and California.

*R. dominica* is considered the most serious pest of stored wheat and rice due to its ability to cause severe damage to sound kernels resulting in substantial economic losses (Hagstrum et al. 2012). The most recent research on phosphine resistance in North American *R. dominica* was conducted in Oklahoma by Opit et al. (2012). Similarly, the only North American work on the occurrence of strong- and weak-resistant phenotypes of *R. dominica* was also done by Opit et al. (2012), and occurrence of these resistance phenotypes has not been documented elsewhere in North America. The objective of the research reported below was to determine the frequency of phosphine resistance in geographically separated populations of *R. dominica* across the United States and Canada using a standard discriminating dose bioassay. Additional work was conducted to ascertain the presence of weak- or strong-resistant beetles within some populations and to characterize these populations using a comparative dose–response assay

# Materials and methods

## Field Collection of *R. dominica* and their Laboratory Maintenance

Insects were live-trapped at 33 collection sites in the United States and Canada using Lindgren multiple-funnel traps baited with lures containing synthetic *R. dominica* aggregation pheromones 'Dominicalure I and II', as described in the study by Edde et al. (2005). For one additional collection site, we received beetles that were not trapped but rather collected from grain debris at a commercial mill and sent to us for testing (Table 2-1, Population 8). While all samples in Kansas and Canada were collected by me, the samples from other parts of North America were collected by cooperating scientists, grain managers, or commercial pest control operators to whom I sent traps, lures, and instructions for handling and return shipping of live specimens back to us. Adult beetles from each location were accumulated in 473-ml Ball wide mouth Mason jars (Hearthmark, LLC, Fishers, IN), closed with a ring-lid equipped with a metal screen and filter paper to prevent the adults from escaping the jar but allow air and moisture exchange. Beetles were held on a mixture of 95% whole wheat kernels and 5% wheat flour, in a growth chamber kept at constant regimes of 28°C, 65% relative humidity (RH) and a photoperiod of 16:8 (L:D) h until used in a fumigation assay. Adults collected from each field site were cultured for up to 6 weeks prior to their use in bioassays, after which progeny reared from the holding jars were retained for additional studies (dose–response experiments described below).

## FAO Discriminating Concentration Bioassay for Determining Resistance

The frequency of phosphine-resistant adults in samples from *R. dominica* field populations was determined using the Food and Agriculture Organization (FAO) Method No. 16 (Food and Agriculture Organization 1975) with the same methods used by Opit et al. (2012), and hereafter

referred to as the ‘FAO assay’. The FAO assay relies on a 20-h fumigation at 25°C in a gas-tight chamber with 20 ppm (0.03 mg/L) of phosphine followed by a 14-d holding period after which the numbers of living and dead beetles were counted. Briefly, we used a fumigation chamber that was a 3.8-liter gas-tight glass jar with a gas-tight screw-on metal lid in which we placed a group of 4-dram ventilated glass shell vials (Kimble Chase Life Science and Research Products, LLC, Vineland, NJ) containing adult beetles for testing (as per methods used by Opit et al. 2012 and Cato et al. 2017). Each of the shell vials contained approximately 500 mg of cracked wheat as food for about 20 beetles during the whole period of the bioassay from fumigation through recovery. For each population sampled from a particular field location, three fumigation jars containing one or more vials with insects from that location were set up as three replicates. The number of insects assayed in each jar varied based on the numbers collected during the sampling or received at the laboratory. Each jar in all these FAO assays also contained adults from our phosphine-susceptible strain, which originated from a reference population maintained at the United States Department of Agriculture (USDA), Center for Grain and Animal Health Research, Manhattan, Kansas, so that we could confirm that each jar had a concentration of gas that could kill susceptible *R. dominica*.

All fumigations were conducted in a growth chamber kept at 25°C and with 50 µl of water deposited on the bottom of each sealed jar to achieve about 70% RH by the end of the exposure period. Groups of insects from different locations were placed together in one jar at the same time for fumigation. Phosphine gas was from a pressurized cylinder of 10,000 ppm (1% in nitrogen) purchased from Linweld (Lincoln, NE). Within 30 min of starting a fumigation assay, a small volume of the 10,000 ppm gas was transferred from the cylinder into an evacuated gas tight Tedlar PVF film bag (CEL Scientific Corporation, Cerritos, CA) for safe transfer of gas to

use in the fumigation chambers. A volume of 11.25 ml of the 10,000 ppm phosphine was injected into a sealed fumigation chamber using a Hamilton 25 ml, Model 1025 TLL (Reno, NV) gas tight syringe immediately after removing an equal amount of air from the chamber, to then produce an atmosphere of 20 ppm of phosphine. The concentration of phosphine in each of the jars was assessed at the beginning and end of the 20-h fumigation period using quantitative gas chromatography–flame photometric detection (GC–FPD) with the method described below. The average between the starting concentration and the ending concentration during the 20-h fumigation period was considered as the designated concentration for a given jar. Replicate fumigation jars used in our FAO assays had measured phosphine concentrations between 17 and 22 ppm and thus were within the range deemed appropriate by FAO (1975; Appendix 5) for discriminating doses to test for resistance to phosphine in *R. dominica*. At the end of the phosphine exposure, each jar was opened and ventilated for 30 min under a fume hood, the insects were removed from the fumigation jars, and returned to the growth chamber in their vials with cracked grain and left for 14 d to allow for recovery or delayed mortality. Any live individuals counted at the end of these assays were considered resistant to phosphine. Numbers of dead and living beetles were pooled for the three replicate FAO assay jars for a given location to derive the estimated frequency of phosphine resistance for that population. Map locations by geographic coordinates in North America for the populations sampled were used to conduct regression analyses of resistance frequencies as a function of geographic location, and these were analyzed with R version 3.2.0 (R Core Team 2015).

**Quantitative Analysis of Phosphine Concentrations with GC**

Analysis and quantification of phosphine concentration in each fumigation chamber was assessed using a Shimadzu GC-17A (Shimadzu Scientific Instruments, Columbia, MO) GC and an integrator-strip chart recorder to report amounts and retention times of analytes, as described previously by Cato et al. (2017). The GC was equipped with a GS-Q column (30 m long × 0.53 mm i.d., 0.25 μm film thickness, J & W Scientific, Folsom, CA) and a FPD set for detecting only phosphorus. The carrier gas used was ultra-high purity helium (Linweld, Lincoln, NE). The concentration of phosphine calculated for each jar relied on first generating an external standard curve prior to each fumigation experiment. The GC detector response measured as the area under the curve for an injected sample of phosphine was converted into the estimated ppm concentration of $PH_3$ in a jar. A 20-ppm phosphine standard was achieved by the dilution of a precise volume of the 1% phosphine into measured volume of air in a CEL Scientific Tedlar PVF film bag. Injections of this standard gas at volumes of 25, 20, 15, 10, and 5 μl were analyzed. The 15-μl injection, which was the size of the headspace sample to be taken from a fumigation jar, was deemed as the 20-ppm concentration and then a standard curve with the linear equation was calculated and used to estimate the ppm concentration of phosphine in a jar from the area integrated under the GC peak for a given phosphine sample.

**Dose–Mortality Bioassays**

The level or strength of resistance within selected populations from this study was ascertained using a dose–response assay for mortality from phosphine, similar to that described by Cato et al. (2017). On the basis of the results from our discriminating dose assays of the 34 populations (Table 1), I selected six populations with low-, medium-, or high-resistant frequencies and for

which I hypothesized could have corresponding resistance phenotypes of susceptible, weak, and strong resistance. Recent research has shown that populations of either *T. castaneum* or *R. dominica* with high frequencies of resistant individuals are more likely to have beetles with strong-resistant phenotypes compared with weak-resistant phenotypes (Opit et al. 2012, Cato et al. 2017). The frequency of the genes responsible for strong resistance in *T. castaneum* is more likely to be found in populations displaying high-resistant frequency and also strong-resistant phenotypes (Chen et al. 2015). These included two susceptible populations (USDA and Starbuck) found to have a resistance frequency of 0%, two populations found with low-to-moderate frequencies of resistance frequencies (≤55%) (Junction City 2 and Wamego) and two populations were found to have ≥80% frequency of resistance (Minneapolis and Belle Glade). After field-collected beetles from these populations were used in the FAO assays, the grain they had been maintained on prior to FAO testing was then cultured for a new generation of adults. Those $F_1$ adults were subsequently re-cultured for two or more additional generations using several colony jars to provide upwards of 1,500 or more beetles per populations for the dose-mortality experiments. Concentration-mortality data for all six populations were generated by exposing them to an incremental range of phosphine concentrations over a fumigation period of 20 h. The aim was to predict the lethal concentrations (LCs) required for achieving mortality of 50% and 99% of the tested individuals in each of the populations. A minimum of 50 adult insects in vials were placed in fumigation jars replicated three times for a particular target concentration and held at 25°C during a 20-h exposure. After ventilation, the jars were then maintained at 27.5°C and 70% RH following fumigation. The phosphine concentrations targeted against the presumed susceptible populations were 2, 4, 6, 10, and12 ppm, the presumed weak-resistant populations were tested at concentrations including 5, 10, 25, 50, 100, 120, 150, 180, and 200

ppm, while the populations we suspected to have the strong-resistant phenotype were tested with target concentrations of 5, 50, 100, 300, 500, 700, 750, 850, 950, and 1000 ppm. Recorded average concentrations quantified for fumigation jars used in these experiments ranged from 1.7 ppm tested against susceptible populations up to 1,585.0 ppm of phosphine for the strong-resistant populations. After the 20-h fumigation period, the insects were removed from the jars and given a 14-d recovery period in the growth chamber. On the basis of the concentrations determined by quantitative GC analysis, each concentration was used as a separate mortality data point, with one targeted concentration yielding three unique concentrations with its corresponding mortality for regression. Using PROC PROBIT from SAS version 9 (SAS 2002), the LCs were estimated from a Probit regression analysis. Comparison among populations for differences in their level of resistance were made after computing resistant ratios based on the $LC_{50}$ value for the population of interest divided by the $LC_{50}$ of the laboratory-susceptible strain, referred to as the $RR_{50}$.

## Results

Table 2-1 shows the frequency of phosphine resistance in 34 North American *R. dominica* populations after evaluation using the FAO bioassay. I collected adult beetles from 34 geographically separate locations. Of the 34 populations, 33 were of beetles collected from pheromone traps, while beetles from just the one population from Belle Glade, FL were collected as adults and immatures in samples of infested grain and grain debris at that location. In this case, I was able to rear adults from the field material in addition to adults present at the time of the collection and use these directly for FAO assays. For trapping of other populations, I deployed a single pheromone trap at each location for the time periods ranging from 2 to 8 weeks and live beetles in traps were brought to the laboratory or shipped to us by cooperators on a weekly basis. Trapped beetles were accumulated in jars of diet in our laboratory and then used in FAO assays after trapping was completed. I had hoped to meet the FAO assay guidelines for testing a total of 100 insects divided between two replicate fumigations, but in most cases, I did not accumulate over 100 beetles. Collections at the Parlier (1) and Williams locations in California had less than 10 adults, and these were reared in the laboratory to yield a few more progeny to allow us to test 30 for each of these sites.

Out of the 34 populations of *R. dominica* assessed, only the Carnduff and Starbuck populations, both from Canada, had no resistant (surviving) beetles following the assays, with all beetles in the collections killed by the discriminating dose of 20 ppm of phosphine for 20 h. Generally, we recorded higher frequencies of resistance in US populations compared with populations from Canada. The frequency of resistant individuals in *R. dominica* populations from the United States ranged from 3 to 97%, whereas those from Canada ranged from 4 to 68% (Table 1). Kansas and California had more populations, 16 and 6 respectively, tested for

phosphine resistance compared with the other states and provinces. The bias in geographic locations of the populations I studied is attributed to where I had permission granted by property and/or business owners who allowed me to set up and service traps. Despite these collection biases, our regression analyses found weak, but significant geographical trends in the resistant frequencies decreasing as a function of increased latitudes going north, with an $R^2 = 0.38$ and $P < 0.01$, and increasing resistance frequencies with increased longitude from east to west, with $R^2 = 0.16$ and $P = 0.01$ 7.

The levels of resistance characterized from dose-mortality experiments are reported in Table 2-2. Six populations presumed to represent susceptible, weak-, and strong-resistant phenotypes were tested at different phosphine concentrations to yield mortality data points (Figure 2-1). On the basis of these dose-mortality experiments, the concentrations of phosphine required to kill 50 and 99% of the laboratory-susceptible *R. dominica* strain were compared with the five field collected populations (Table 2-2). The resistance ratio factors, $RR_{50}$, were subsequently calculated using the estimated $LC_{50}$ values. The $RR_{50}$ values for Belle Glade, FL and Minneapolis, KS were 595- and 100-fold, respectively, compared with the susceptible, which suggests they can be classified as having beetles with strong-resistant phenotypes, The Junction City 2 and Wamego populations in Kansas, which represented weak-resistant phenotypes, were recorded with 5- and 9-fold resistance levels based on $RR_{50}$, respectively. Beetles from the Starbuck, Manitoba population, which had 0% resistance in the discriminating dose assays (Table 2-1) can be classified as having phosphine-susceptible phenotypes like that of our laboratory-susceptible strain due to its $RR_{50}$ being 1.2 (Table 2-2). A visual representation of the phosphine-resistant phenotypes of these six populations can be seen in the concentration-mortality plots in Figure 2-2 in which the proportion of adults killed are plotted against the tested

phosphine concentrations reported on a log-10 scale for the x-axis. I was unable to achieve 100% mortality for any concentrations tested on the two strong-resistant populations, Minneapolis, KS and Belle Glade, FL, with no mortality from the former being greater than 80% and none from the latter being greater than 40%.

## Discussion

This study of phosphine resistance in *R. dominica* involved more extensive sampling than previous work in collecting field insects to assess the frequency and levels of resistance in several geographical locations across the US and Canada. Results from the current study showed much higher frequencies of phosphine-resistant insects in the US compared to the first global geographical study. Only three populations of *R. dominica* in North America were surveyed by Champ and Dyte (1976; Table F11 on p. 134 and Fig. F17 on p. 138) and all three were susceptible to phosphine with no resistance detected at that time. The same study reported resistance occurring at frequencies ranging from 11 to 57% in *R. dominica* across the remaining seven continental regions.

The current study found that 32 out of 34 (94%) populations sampled in five states in the United States and three provinces in Canada have individuals that were resistant to phosphine. To put this into perspective, all 24 populations tested for phosphine resistance in the United States were resistant. This confirms the widespread development and/or spread of phosphine resistance in North America covering several states and provinces, and these data complement recent findings in Oklahoma of all five *R. dominica* populations being resistant (Opit et al. 2012). The two populations that showed phosphine susceptibility were collected from Canada at the Carnduff and Starbuck sites. Susceptibility in these two Canadian locations may be due to the absence of resistant genes, or if the resistance genes were present they may have been at very low frequencies such that any resulting resistance phenotypes were undetectable from our sampling. In addition, the low-resistant frequencies we found in Canada could be attributed to the low selection pressure for resistance as most grain facilities in Canada, particularly west of Ontario, conduct few or no phosphine fumigations in any given year (unpublished reports to the

Canadian Grain Commission). The frequencies of resistant individuals in the other Canadian populations were much lower compared with the frequencies in the US populations. Of the eight Canadian populations sampled, only two had resistance frequencies greater than 50%, whereas in the United States 21 of the 24 populations sampled had resistance frequencies greater than 50%. Although we found phosphine resistance to be common among populations of *R. dominica* in North America, phosphine resistance was not commonly found in surveys of grain insects conducted in the 1980s. One study looked at the frequencies of phosphine resistance in one beetle species, *T. castaneum*, and in two moth species, *Cadra cuatella* (Walker) and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae), infesting stored peanuts in Florida, Alabama, and Georgia and found that less than half of the populations surveyed were resistant, and of those the frequencies of resistance were low (Zettler et al. 1989). Another project studied beetle populations infesting stored wheat in Oklahoma and found that *T. castaneum* had just 1 out of 7 populations (14%) with resistance, and 8 out of 21 populations (38%) of *R. dominica*to be resistant (Zettler and Cuperus 1990). Opit et al. 2012 sampled insects from some of the same locations in Oklahoma originally sampled by Zettler and Cuperus (1990) and found that five out of five *R. dominica* populations (100%) and eight out of nine *T. castaneum* populations (89%) were resistant to phosphine. These results indicate that resistance frequencies have increased for those two species between 1990 and 2012 in Oklahoma.

Our recent work on a North American survey of phosphine resistance in *T. castaneum* reported just 12 out of 25 populations (48%) had detectable resistance, with none of the 4 Canadian populations sampled from that species having any resistance (Cato et al. 2017). Several of the locations sampled by Cato et al. (2017) for *T. castaneum* were the same for those

sampled here for *R. dominica*, thus it is likely that selection pressure from fumigation practices were similar for both species at those locations.

California and Kansas were the two states that had the most sites tested for insects resistant to phosphine. All the sites in these states showed varying frequencies of phosphine-resistant insects with five sites showing levels ≥80%. California is known for high production of rice, whereas Kansas is a large producer of wheat. Information from colleagues who trapped *R. dominica* for us in California and our own experience with the collection sites in Kansas concur that phosphine is likely the only fumigant used in the control of stored grain insects in both states. The high use of phosphine can serve as an increased selection pressure for the resistance alleles, and the ability of *R. dominica* to fly over long distances might have contributed to the spread of these alleles in these areas accounting for the frequencies observed (Mahroof et al. 2010, Ridley et al. 2016). Beetles sampled from Colusa in California had a resistance frequency of only 3%, which could be due to very low frequencies of resistant genes under an environment of regular effective phosphine use, or from minimal selection pressure of resistant genes due to a low use of phosphine in that county. Data from the California Environmental Protection Agency on the use of phosphine in 10 counties known for regular phosphine fumigations showed Colusa County recorded one of the lowest amounts of aluminum phosphide and magnesium phosphide usage among those 10 counties from 2005 to 2010 (Gurusinghe 2014). Previous studies have also found occurrences for which a low frequency of phosphine resistance may occur in populations that are very close to resistant populations in the United States (Zettler et al. 1989; Zettler and Cuperus 1990; Opit et al. 2012), but with no reason hypothesized for the lack of area-wide similarity in resistance.

This chapter describes weak and strong forms of resistance in North American *R. dominica.* Collins et al. (2002) were the first to report how different levels of phosphine resistance can occur within a species like *R. dominica*, for which two levels of resistance, weak and strong, can be categorized based on the amount of phosphine required for mortality. In this study, we found that two weak-resistant populations from Kansas, at Wamego and Junction City 2, were 9.5- and 5.2-fold more resistant than our USDA laboratory-susceptible strain based on the resistance ratios calculate from their $LC_{50}$ values. However, the two strongly resistant populations from Minneapolis, KS and Belle Glade, FL were 100.2- and 595.9-fold more resistant than the USDA laboratory-susceptible strain.

Studies in Australia have described how insects exhibiting the typical weak-resistant phenotype are homozygous for resistance alleles at one gene locus, referred to as *rph*1, but that the strong-resistant phenotype requires the beetle to be homozygous for resistance at *rph*1 and to also be homozygous for resistance alleles at a second locus, referred to as *rph*2, and that these two loci apparently work synergistically to express the strong-resistant phenotype (Schlipalius et al. 2012). It is very likely that beetles in both the Minneapolis and Belle Glade populations of *R. dominica* studied here have this key combination of resistant genes that contribute to the high levels of phosphine resistance recorded here.

The gas exposure time and temperature, in addition to an effective gas concentration, when fumigating with phosphine plays a key role in its effectiveness for the most tolerant life stages of a pest species (Hagstrum et al. 2012). The frequency and strength of phosphine resistance in *R. dominica* reported in our study are based on experiments with a 20-h phosphine exposure period at 25°C using adult beetles, which is the standard framework for the FAO assays and the methods allowed me to discriminate resistant from susceptible beetles in a standard,

simple way. I continued to use a 20-h exposure time at 25°C for our dose–mortality studies that characterized weak- and strong-resistant phenotypes based on phosphine concentration alone. Those dose–response experiments clearly distinguished the weak- and strong-resistant phenotypes, but the estimated concentrations needed for 99% mortality should not be considered for practical applications to control infestations in the field, for which 20 h would be impractical and never recommended (Hagstrum et al. 2012). The estimated $LC_{99}$ for the resistant *R. dominica* from Belle Glade, FL was calculated to have an upper fiducial limit of 28,174 ppm during a 20-h fumigation. This is an unrealistically high concentration that could never be achieved in the laboratory or the field using current technology. Opit et al. 2012, who also described strongly resistant populations of *R. dominica*, proposed that such resistant insects cannot be controlled with any practical concentration of phosphine unless insects were held under a critical concentration for ≥3 d. A study carried out by Kaur and Nayak (2015) showed that to achieve complete mortality of resistant populations of *Cryptolestes ferrugineus*, higher gas concentrations and longer exposure periods than those prescribed in the current application label rates for phosphine fumigation would be required. Thus, although we report the levels of resistance in *R. dominica* that are nearly 600 times greater compared with susceptible beetles and possibly requiring an unrealistic level over 20,000 ppm to control 99% of a population, the true challenge posed by beetles with this level of resistance will need further research under conditions more like those in commercial fumigations under field conditions.

My findings of phosphine resistance occurring in nearly all populations of *R. dominica* studied, the relatively high frequency of resistance in those populations, and the finding of many populations likely having insects with the strong-resistant phenotype, point to the challenge pest managers are facing with phosphine resistance. The levels of resistance in the

United States call for the use of a resistance monitoring program that pest managers can use to make decisions about using phosphine or not in a given situation. Australia has been successful in implementing a resistance monitoring program and using the information in regional or local management recommendations (Emery et al. 2011). If a monitoring program determines that resistance is very low or nil at a given site then management strategies such as proper sealing of grain storage structures to prevent leakage of gas, and gas monitoring through treatment to confirm effective concentrations, can be adopted to maintain the efficacy of phosphine for susceptible pests (Emery et al. 2011). Resistance management programs that target pests having a low frequency of phosphine resistance may include proper phosphine fumigation techniques at high concentrations for long hold times, but only when needed at on-farm and commercial storage facilities after preventive methods are exhausted (Cuperus et al. 1993). Persistent phosphine resistance at a site, especially if strong resistance is suspected or if there is any history or suspicion of control failures with phosphine, should lead managers to consider a total cessation of phosphine use and a change to another pesticide with a different mode of action. Other grain fumigants such as sulfuryl fluoride can be very effective against phosphine-resistant insects (Opit et al. 2016).

# References

**Cato, A. J., Elliott, B., Nayak, M. K., and T. W. Phillips. 2017.** Geographic variation in phosphine resistance among North American populations of the red flour beetle. J. Econ. Entomol. 110: 1359–1365.

**Champ, B. R., and C. E. Dyte. 1976.** Report of the FAO global survey of pesticide susceptibility of stored grain pests. FAO Plant Production and Protection Series, 5, FAO, Rome, Italy.

**Chaudhry, M. Q. 2000.** Phosphine resistance. Pestici. Outlook 11: 88–91.

**Chen, Z., Schlipalius D., Opit G., Subramanyam B., and T. W. Phillips. 2015.** Diagnostic molecular markers for phosphine resistance in U.S. populations of *Tribolium castaneum* and *Rhyzopertha dominica*. PLoS One 10: e0121343.

**Collins, P. J., Daglish G. J., Bengston M., Lambkin T. M., and H. Pavic. 2002.** Genetics of resistance to phosphine in *Rhyzopertha dominica* (Coleoptera: Bostrichidae). J. Econ. Entomol. 95: 862–869.

**Cuperus, G., Noyes R. T., Fargo W. S., Kenkel P., Criswell J. T., and K. Anderson. 1993.** Reducing pesticide use in wheat postharvest systems. Cereal Food World 38: 199–203.

**Edde, P. A., Phillips T. W., and M. D. Toews. 2005.** Responses of *Rhyzopertha dominica* (Coleoptera: Bostrichidae) to its aggregation pheromones as influenced by trap design, trap height and habitat. Environ. Entomol. 34: 1549–1557.

**Emery, R. N., Nayak M. K., and J. C. Holloway. 2011.** Lessons learned from phosphine resistance monitoring in Australia. Stewart Postharvest. 7: 1–8.

**Food and Agriculture Organization. 1975.** Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides – Tentative method for adults of

some major pest species of stored cereals, with methyl bromide and phosphine. FAO method no. 16. FAO Plant Prot. Bull. 23:12–25.

**Gurusinghe, P. 2014.** Fumigants: phosphine and phosphine-generating compounds. Risk Characterization Document. California Environmental Protection Agency. Department of Pesticide Regulation. Environmental Monitoring Branch, p. 33, Sacramento, California.

**Hagstrum D. W., Phillips T. W., and G.W. Cuperus. 2012.** Stored product protection. Kansas State University, Manhattan, KS. KSRE Publ; S–156.

**Kaur, R., and M. K. Nayak. 2015.** Developing effective fumigation protocols to manage strongly phosphine-resistant *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae). Pest. Manag. Sci. 71: 1297–1302.

**Kaur, R., Subbarayalu, M., Jagadeesan, R., Daglish, G. J., Nayak, M. K., Naik, H. R., Ramasamy, S., Subramanian, C., Ebert, P. R., and D. I. Schlipalius. 2015.** Phosphine resistance in India is characterized by a dihydrolipoamide dehydrogenase variant that is otherwise unobserved in eukaryotes. Heredity 115: 188–194.

**Lorini, I., Collins P. J., Daglish G. J., Nayak M. K., and H. Pavic. 2007.** Detection and characterization of strong resistance to phosphine in Brazilian *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae). Pest Manag. Sci. 63: 358–364.

**Manivannan S. 2015** Toxicity of phosphine on the developmental stages of rust-red flour beetle, Tribolium castaneum Herbst over a range of concentrations and exposures. Journal of food science and technology J. Food Sci. Technol. 6810-6815.

**Mau YS, Collins PJ, Daglish GJ, Nayak MK and P.R Ebert. 2012**. The rph2 gene is responsible for high level resistance to phosphine in independent field strains of Rhyzopertha dominica. PloS one 7(3): e34027 (2012).

**Mau YS, Collins PJ, Daglish GJ, Nayak MK, Pavic H and P. R. Ebert.** The rph1 gene is a common contributor to the evolution of phosphine resistance in independent field isolates of *Rhyzopertha dominica*. Plos One 2012;7(2):e31541.

**Nayak, M. K., Collins, P. J., Holloway, J. C., Emery, R. N., Pavic, H., and J. Bartlet. 2013.** Strong resistance to phosphine in the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae): its characterisation, a rapid assay for diagnosis and its distribution in Australia. Pest Manag. Sci. 69: 48–53.

**Nayak, M. K., Daglish, G. J., and, T.W. Phillips. 2015.** Managing resistance to chemical treatments in stored products pests. Stewart Postharvest Rev. 11: 1–6.

**Opit, G. P., Phillips T. W., Aikins M. J., and M. M. Hasan. 2012.** Phosphine resistance in *Tribolium castaneum* and *Rhyzopertha dominica* from stored wheat in Oklahoma. J. Econ. Entomol. 105: 1107–1114.

**Opit, G. P., Thoms E., Phillips T. W.,and Payton M. E.. 2016.** Effectiveness of sulfuryl fluoride fumigation for the control of phosphine-resistant grain insects infesting stored wheat. J. Econ. Entomol. 109: 930–941 .

**Ridley, A. W., Hereward J. P., Daglish G. J., Raghu S., and G. A. McCulloch. 2016.** Flight of *Rhyzopertha dominica* (Coleoptera: Bostrichidae)—a spatio-temporal analysis with pheromone trapping and population genetics. J. Econ. Entomol. 109: 2561–2571.

**R Core Team. 2015.** R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. http://www.R-project.org/ (accessed January 2015).

**SAS. 2002.** User's Guide, v. 8. SAS Institute, Cary, NC.

**Schlipalius, D. I.,Valmas, N., Tuck, A. G., Jagadeesan, R., Ma, L., Kaur, R., Goldinger, A., Anderson, C.,Zuryn, S., Kuang, J., et al. 2012.** A core metabolic enzyme mediates resistance to phosphine gas. Science 338: 807–810.

**Zettler, J. L., and G. W. Cuperus 1990.** Pesticide resistance in *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae) in wheat. J. Econ. Entomol. 83: 1677–1681.

**Zettler, J. L., Halliday W. R., and F. H. Arthur. 1989.** Phosphine resistance in insects infesting stored peanuts in the southeastern United States. J. Econ. Entomol. 82: 1508–1511.

**Table 2-1. Percentage of phosphine resistant individuals in North America populations of *Rhyzopertha dominica* using the FAO discriminating dose bioassay**

| Population | Country | State/Province | City[a] | % Resistant (*n*) |
|---|---|---|---|---|
| | USA | | | |
| 1 | | Alabama | Uniontown | 89 (90) |
| 2 | USA | California | Parlier (1) * | 97 (30) |
| 3 | | | Parlier (2) | 72 (90) |
| 4 | | | Williams* | 53 (30) |
| 5 | | | Colusa | 3 (60) |
| 6 | | | Princeton | 71 (31) |
| 7 | | | Biggs | 7 (150) |
| 8 | USA | Florida | Belle Glade | 87 (90) |
| 9 | USA | Kansas | Manhattan (1) | 66 (59) |
| 10 | | | Manhattan (2) | 77(150) |
| 11 | | | Manhattan (3) | 75 (60) |
| 12 | | | Manhattan (4) | 71 (90) |
| 13 | | | Manhattan (5) | 78 (90) |
| 14 | | | Manhattan (6) | 51 (50) |
| 15 | | | Manhattan (7) | 66 (90) |
| 16 | | | Manhattan (8) | 48 (40) |
| 17 | | | Abilene (1) | 93 (30) |
| 18 | | | Abilene (2) | 90 (20) |
| 19 | | | Minneapolis | 93 (60) |
| 20 | | | Clifton | 80 (90) |
| 21 | | | Junction City (1) | 57 (30) |
| 22 | | | Junction City (2) | 50 (40) |
| 23 | | | Garden City | 74 (39) |
| 24 | | | Wamego | 54 (149) |
| 25 | USA | Texas | Victoria | 67 (60) |
| 26 | | | Burleson | 87 (90) |
| 27 | Canada | Alberta | Lethbridge | 4 (90) |
| 28 | | | Stirling | 14 (90) |
| 29 | | | Bow Island | 38 (50) |
| 30 | Canada | Saskatchewan | Carnduff | 0 (90) |
| 31 | | | Coronach | 19 (90) |
| 32 | | | Weyburn | 68 (50) |
| 33 | | | Indian Head | 62 (100) |
| 34 | Canada | Manitoba | Starbuck | 0 (90) |

[a]Populations denoted with (*) were tested after being in the laboratory for > 2 generations. City names followed by parenthetical numbers refer to the collection sites for a given numbered population nearby to those cities and with the approximate locations shown in Figure 1.

**Table 2-2. Probit analysis for mortality responses of adult *Rhyzopertha dominica* from six selected populations to varying concentrations of phosphine during 20 hr exposure at 25 °C**

| Population | $LC_{50}$ (95% FL) ppm | $LC_{99}$ (95% FL) ppm | Slope | Y-Intercept | $RR_{50}$ | $\chi 2$ (df) | P-value[a] |
|---|---|---|---|---|---|---|---|
| USDA | 3.46 (3.23-3.72) | 9.511 (8.12-11.76) | 5.30 | -2.86 | 1.00 | 10.12 (7) | 0.18 |
| Starbuck | 4.23 (3.94-4.54) | 11.18 (9.71-13.47) | 5.51 | -3.46 | 1.22 | 16.44 (13) | 0.23 |
| Wamego | 33.09 (29.46-37.05) | 503.54 (380.25-711.19) | 1.97 | -2.99 | 9.56 | 21.95(16) | 0.15 |
| Junction city 2 | 20.00 (16.79-23.31) | 517.32 (334.91-943.51) | 0.71 | -2.14 | 5.22 | 14.06 (11) | 0.23 |
| Minneapolis | 346.62 (272.99-430.89) | 59681 (27112-186465) | 0.45 | -2.64 | 100.18 | 15.06 (12) | 0.23 |
| Belle Glade | 2062.00 (1748.00-2957.00) | 8086 (4705.00-28174.00) | 0.39 | -2.60 | 595.95 | 5.25 (7) | 0.64 |

[a]The mortality data collected using different concentrations of $PH_3$ are not significantly different from what the Probit regression model would predict if P>0.05 for a given population.

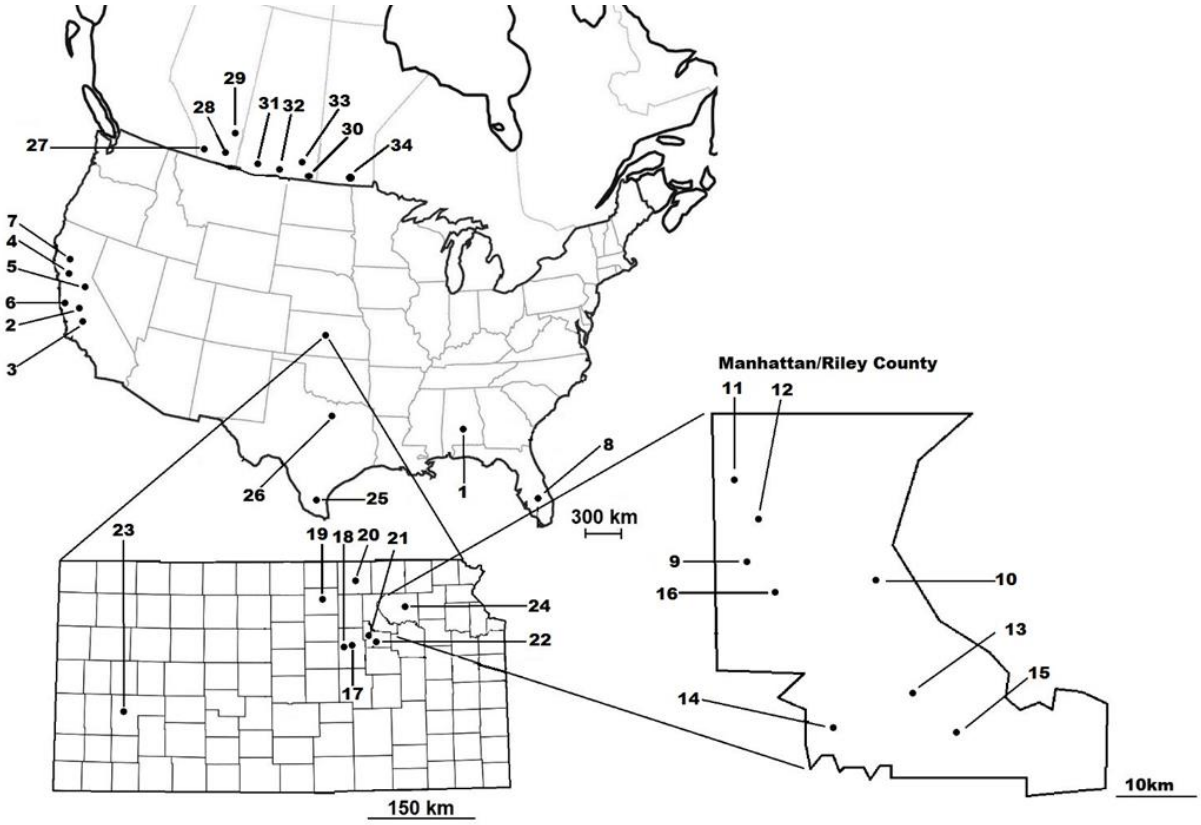


**Figure 2-1. North America map showing approximate geographic locations of the thirty-four populations of *Rhyzopertha dominica* tested for phosphine resistance. All locations and scale are approximate**

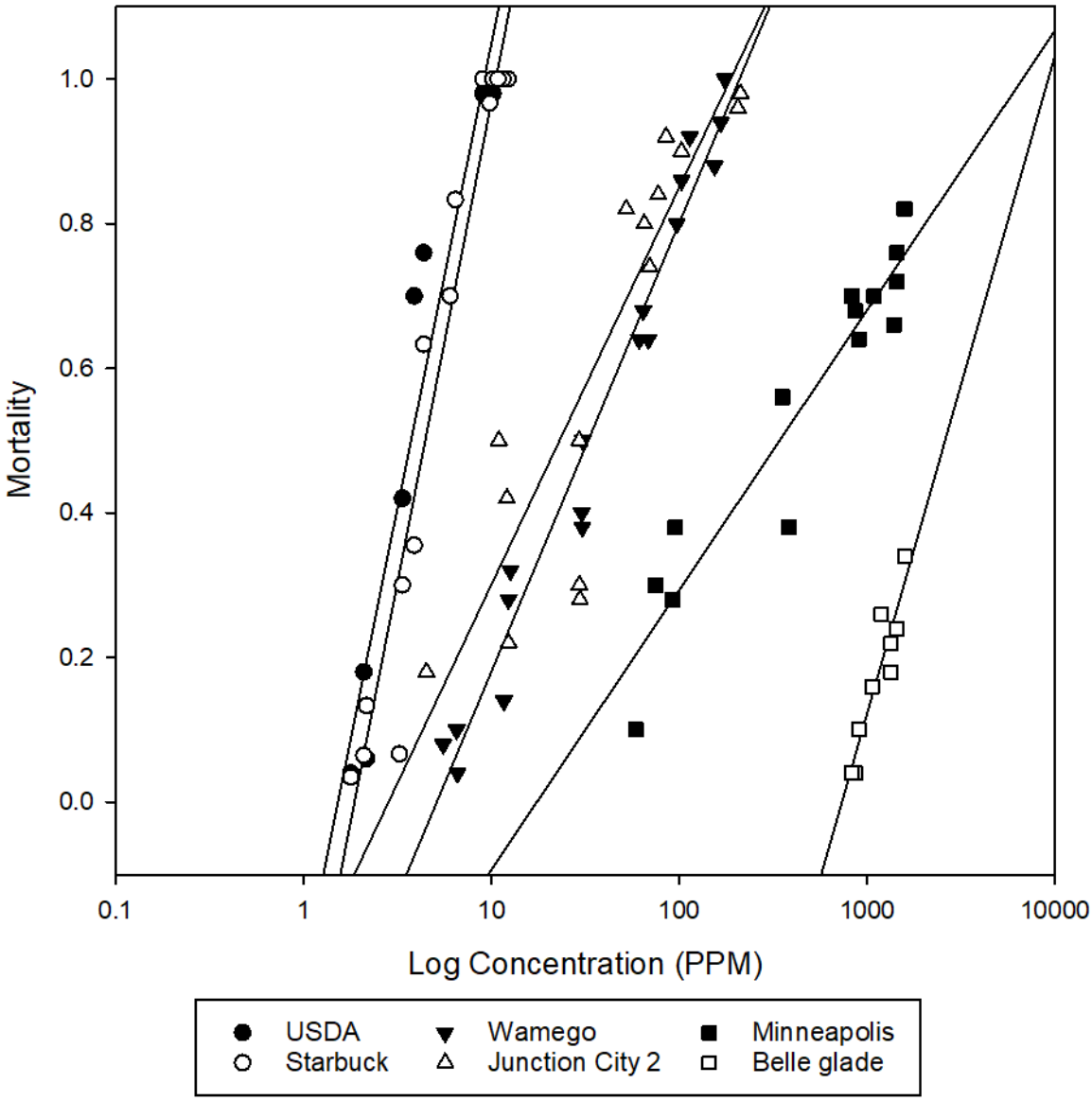


**Figure 2-2. Dose-response plots for six populations showing designated phenotypes of phosphine-susceptible (USDA and Starbuck), weak resistant (Wamego and Junction City 2) and strong-resistant (Minneapolis and Belle Glade) using Probit analysis.**

# Chapter 3 - High dose strategies for controlling phosphine-resistant populations of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae)

## Abstract

Chapter 2 of this dissertation determined that 32 out of 34 populations of *Rhyzopertha dominica* in North America had frequencies of resistance ranging from 4-97% after testing adult beetles with a discriminating dose assay. Populations in Kansas and Florida had beetles with the "strong resistance" phenotype, which is characterized by having resistant factors based on $LC_{50}$ values that were 100- and 595-fold higher than those of a susceptible strain in comparison to "weak resistant" beetles with resistance factors close to 10-fold. The objective of this study therefore, was to determine the minimum gas concentrations and days of exposure at 25$^{o}$C needed to kill all life stages of beetles from these two populations. Such laboratory results can be used to recommend gas concentrations and hold times needed to effectively control populations of strongly resistant populations of *R. dominica*. A dose response assay estimated that phosphine fumigation done at 48 hours using doses of 730-870 ppm could control all resistant adult *R. dominica*. The study also found phosphine concentrations ranging from 400 - 800 ppm phosphine for 96 hours completely killed mixed life stage colonies of strongly resistant lesser grain borers from the two populations studied. Additionally, fumigations done beyond 4 days at phosphine concentrations between 450-700ppm controlled all phosphine resistant lesser grain populations identified from our previous work. The fumigation protocols developed in this study could be used in recommending changes to the currently registered rates of phosphine in United States towards management of strongly resistant *R. dominica* populations.

# Introduction

The United States is a world leader in wheat production. Due to high amount of wheat production comes the problem of losses associated with stored-wheat insect pests. The main method used for controlling insect infestations in stored grain throughout the world is fumigation with toxic gases. When properly applied, fumigants deliver a high level of mortality and leave no chemical residue on grain or food to pose a health concern (Phillips et al 2012). Phosphine is the most commonly used fumigant to control many stored product insect pests worldwide. Phosphine is relatively inexpensive, easy to apply, and can be used for a wide range of storage structures and commodities. As with most fumigants, this toxin leaves little or no chemical residue making it a safe fumigant for use on most food commodities. (Davis, 2003).

A major threat to the continuous use of phosphine is the development of resistance in several key stored grain pests. In 1972 and 1973 a global survey was undertaken by the Food and Agriculture Organization (FAO) of the United Nations to assess the presence or absence of insects resistant to phosphine. The results of the survey showed that about 10 % of the collected populations contained phosphine resistant individuals (Champ and Dyte, 1976). Since then, studies in countries such as Morocco (Benhalima et al. 2004), Brazil (Athie and Mills, 2005), Vietnam (Bui, 1999), China (Yan et al. 2004), Australia (Collins, 1998; Bengston et al.1999; Valmas and Ebert, 2006) and Pakistan (Ahmedani et al. 2006) have reported high levels of phosphine resistance in different insect species. Currently, 15 species of insects are known to have developed resistance to phosphine (Champ and Dyte 1976, Chaudhry 2000, Nayak et al. 2003), and resistance in these species is showing an expanded distribution within many of these species (Benhalima et al. 2004). Low levels of resistance to phosphine in USA stored-grain insects collected in Oklahoma were first reported in the 1980s (Zettler and Cuperus 1990). Thirty

years later, high frequencies of resistance were found in *Rhyzopertha dominica* (F.) and *Tribolium castaneum* in Oklahoma (Opit et al. 2012a) and in several other locations in the US and Canada (Cato et al 2017; Afful et al 2017).

Phosphine resistance studies conducted in Australia over the past decade have identified two resistance phenotypes for *T. castaneum* and *R. dominica* beetles: a strong and a weak resistance phenotype (Collins et al 2005, Schlipalius et al 2002). The "weak" resistance phenotype may require phosphine concentrations of 10-fold to 50-fold greater than those needed to kill susceptible beetles, and beetles with the "strong" resistance phenotype may require 100-fold or greater concentrations relative to susceptible beetles (Nayak et al 2015). Genetic analysis of these insect species from Australia identified two loci conferring strong resistance in both *R. dominica* and *T. castaneum* (Jagadeesan et al 2012; Mau et al 2012a; Mau et al 2012b). The first, rph1 (tc-rph1 for *T. castaneum*), is responsible for weak resistance, whereas the second, rph2, acts synergistically with rph1 to confer strong resistance (Schlipalius et al 2002).

Currently, there are few practical alternatives to phosphine to mitigate grain pest infestations, thus there is a need to preserve effective use of phosphine until one or more suitable alternatives are developed. Phosphine can be used to control resistant populations in some insect species by applying increases in both the gas concentration and the exposure time above those usually recommended (Manivannan, 2015). Additionally, several studies have shown that the manipulation of concentration and exposure periods can be utilized to manage strong resistant populations (Bell, 2015; Daglish et al 2002; Ho and Wink 1995; Winks, 1984).

A study by Nayak et al (2015) to develop robust fumigation protocols that could be used in a range of practical grain storage situations to control strongly resistant *Cryptolestes. ferrugineus* populations by manipulating concentrations and temperature was carried out in

Australia. The study established fumigation protocols in the laboratory for three phosphine concentrations (1.0, 1.5 and 2.0 mg $L^{-1}$) and three temperatures (25, 30 and 35 °C) for the control of all life stages of strongly resistant *C. ferrugineus*. Findings of that study stipulated that a successful control of resistant *C. ferrugineus* can be achieved using shorter fumigation periods at elevated grain temperatures, irrespective of the concentration used.

*R. dominica* is one of the most injurious pests known to attack stored grain (Chittenden, 1911). Both the larvae and adults cause damage to stored grain by boring irregularly shaped holes into whole, undamaged kernels and the larvae, immature stages, may develop inside the grain. This results in a reduction in profits for the grain owner from damaged kernels caused by the activity of the lesser grain borer. Chapter 2 of the dissertation determined that 32 out of 34 populations of *R. dominica* studied in North America had frequencies of resistance ranging from 4-97% after testing adult beetles with a discriminating dose assay. Populations in Kansas and Florida had beetles with the "strong resistance" phenotype, which is characterized by having resistant factors based on $LC_{50}$ values that were 100- and 595-fold higher than those of a susceptible strain in comparison to "weak resistant" beetles with resistance factors close to 10-fold.

The goal of the of the present study was to determine the minimum gas concentrations and number of days of exposure needed to effectively control all life stages of strong phosphine resistant populations of *R. dominica* in laboratory cultures, and to propose recommendation for use of such application methods for phosphine resistance management of this serious grain pests. Specific objectives for our experiments were:

1. Characterize strong resistance in previously studied *R. dominica* populations with dose mortality experiments of adults exposed for 48 h rather than for 20 h.

2. Evaluate efficacy of both high and low concentrations of phosphine applied to mixed life stage laboratory cultures for 48 h to representative weak resistant and strong resistant populations.
3. Determine the number of days of exposure needed to control representative strong resistance populations exposed to either high or low concentrations of phosphine.

# Materials and methods

## *Rhyzopertha dominica* Rearing

Stock cultures of insects used for this study originated from field-collections and had been propagated in the lab for two years under standard conditions (Afful et al. 2017). The lab susceptible reference strain, referred to as USDA, originated from a reference population maintained at the United States Department of Agriculture (USDA), Center for Grain and Animal Health Research, Manhattan, Kansas, for over 40 years. Chapter 2 of this thesis evaluated five populations for weak vs strong levels of resistance using $LC_{50}$ values from dose-mortality experiments (Afful et al. 2017). Laboratory cultures for these same five field populations and the USDA reference strain were maintained as described previously. Adults 2-4 weeks old used for the initial dose mortality assays at 48 h exposures (see below) were re-taken directly from these established laboratory colonies. All other experiments reported here used mixed life stage cultures in 100-ml ventilated glass jars on a mixture of ~30g of whole wheat kernels (*Tritichum aestivum*) and ~10 mg wheat flour. Fifty mixed sex adults from each population were added to the food and kept in a growth chamber at constant regimes of 28°C, 65% relative humidity (RH) and a photoperiod of 16:8 (L:D) h for six weeks to have a mixture of all life stages for fumigation. *R. dominica* has long-lived adults (Edde and Phillips, 2006b) and cultures held for six weeks are expected to have the initial 50 adults, plus eggs laid daily by those initial females, all other immature life stages developing in the grain, and any new F1 adults that may emerge by the end of the six weeks. Thus, each mixed life stage culture jar simulates a small population and allows our fumigation experiments to act on killing all life stages that represent a range of phosphine susceptibility.

**Fumigation Protocol**

Fumigations were done following methods in chapter 2 with some modifications. All fumigations were conducted in a growth chamber kept at 25°C and RH of 70%. Phosphine gas was from a pressurized cylinder of 10,000 ppm (1% in nitrogen), purchased from Linweld (Lincoln, NE), that was diluted to a needed concentration at the time of an experiment. Fumigation chambers were 3.8-liter jars, airtight and equipped with an injection port in the center of the metal screw-on lid. This port was fitted with a rubber injection septum that was used for the introduction and sampling of the fumigant (Opit et al. 2012). The concentration of phosphine in each of the jars was assessed at the beginning and end of each fumigation period outlined below using a quantitative gas chromatography–flame photometric detection (GC–FPD; see below). The average between the starting concentration and the ending concentration during each fumigation period was considered as the designated concentration for a given jar.

**Quantitative GC Analysis of Phosphine Concentrations**

In analyzing and quantifying the exact concentrations needed for each fumigation I followed methods used recently by Cato et al (2017) with some slight modifications. Each fumigation chamber containing phosphine was analyzed and quantified using a Shimadzu GC-17A (Shimadzu Scientific Instruments, Columbia, MO) GC and an integrator-strip chart recorder to report amounts and retention times of analytes (Cato et al 2017). The GC was equipped with a GS-Q column (30 m long × 0.53 mm i.d., 0.25 μm film thickness; J & W Scientific, Folsom, CA) and a FPD set for detecting only phosphorus. The carrier gas used was ultra-high purity helium (Linweld, Lincoln, NE). To determine the phosphine concentration for each jar, an external standard curve was generated. The GC detector response measured as the area under the

curve for an injected sample of phosphine was converted into the estimated ppm concentration of $PH_3$ in a jar. A 500-ppm phosphine standard was achieved by the dilution of a carefully measured volume of the 1% phosphine mixed into a carefully measured volume of air in a CEL Scientific Tedlar PVF film bag. Injections of this standard gas at volumes of 25, 20, 15, 10, and 5 µl were analyzed. The 15-µl injection, which was the size of the headspace samples to be taken from a, experimental fumigation jar, was deemed as the 500-ppm concentration, and then injections of 833, 667, 500, 333 and 167 ppm were made and peak area measurements used to make a standard curve from which the linear equation was calculated and used to estimate the ppm concentration of phosphine in an experimental jar from the area integrated under the GC peak for a given phosphine sample.

**Dose mortality assays of adults**

In the previous chapter I estimated the $LC_{99}$ concentrations for strongly-resistant *R. dominica* based on 20 h fumigation, which is not typical for fumigation in practice (Afful et al 2017). I did a dose-response study to estimate $LC_{99}$ but used a 48 h exposure period that is typical of many commercial fumigations with the intention of determining a starting dose I could use on mixed life cultures of the Belle Glade, FL and Minneapolis, KS. Concentration-mortality data for Belle Glade and Minneapolis populations were generated by exposing adults to an incremental range of phosphine concentrations for 48 h. A similar protocol was followed for the USDA lab strain with the aim of predicting the lethal concentrations (LCs) required for achieving mortality of 50% and 99% of the tested individuals in each of the populations. A minimum of 50 adult insects in vials were placed in fumigation jars and three separate jars were set up at each target concentration and held at 25°C for the 48-h exposure. After ventilation the jars were then

maintained at 27.5°C and 70% RH following fumigation. The phosphine concentrations targeted against the USDA lab susceptible population were 0.5, 1, 1.5, and 2 ppm, while the two populations with the strong-resistant phenotype were tested with target concentrations of 50, 100, 200, 300, 400, 500, 650, 750, 850, 900, and 1000 ppm. Recorded average concentrations quantified for fumigation jars used in these experiments ranged from 0.58 ppm tested against susceptible populations up to 994.07 ppm of phosphine for the strong-resistant populations. After fumigation the insects were removed from the jars and given a 14-d recovery period in the growth chamber. Using PROC PROBIT from SAS version 9 (SAS 2002), the LCs were estimated from a Probit regression analysis. Comparison among populations for differences in their level of resistance were made after computing resistant ratios based on the $LC_{50}$ value for the population of interest divided by the $LC_{50}$ of the laboratory-susceptible strain, referred to as the $RR_{50}$.

**Effect of low and high phosphine concentrations on mortality of *R. dominica* mixed life stages**

Based on the results from the 48 h dose response assay of adults I decided to test the effect of phosphine concentrations on mixed life stages on all six populations from chapter 2 (Afful et al 2017). Using target concentrations of 200 and 1000 ppm, which encompass the ranges of effective concentrations determined from the adult assays at 48 h. Each target concentration was replicated three times with an air control treatment for each of the populations. Recorded average concentrations quantified for each fumigation jar for the target concentration of 200 ppm were 269.25, 297.21 and 282.68 ppm while those targeting 1000 ppm were measured at 774.41, 931.88 and 857.90 ppm. Mixed life stage colonies of each population were obtained using the

rearing protocol outlined above and fumigation was done for 48 hours. After fumigation all adult *R. dominica* were sieved and discarded. Each jar was then assessed weekly for emerged live adults over six weeks for both the treated and untreated jars. The total number of living adult progeny was determined for each jar and the mean values and standard errors calculated both treated and untreated jars of each population.

**Effect of varied phosphine concentrations and varied exposure times on control of strong resistant R. dominica mixed life stage cultures**

The strong resistant populations from Florida and Kansas were not completely killed with the 1000 ppm and 200 ppm concentration for 48 h fumigation respectively. I hypothesized that 100% kill could be achieved if a steady increase in the concentrations and exposure times were tested. Two experiments were conducted to meet this objective.

First, the effect of varied phosphine concentrations on the progeny emergence of *R. dominica* after a 96 h fumigation with different gas concentrations was assessed. Target concentrations were: 150, 300, 450, 600, 750, 900, 1050 and 1200 ppm with two replications (jars) each and two replications with untreated control cultures. Recorded average concentrations quantified for fumigation jars ranged from 150.07 ppm up to 1423.70 ppm. Immediately after the 96-h fumigation all adults were sieved and discarded. The number of adult insects that emerged weekly for six weeks following fumigation, a period that allowed for development of surviving immatures, were counted in both treated and untreated jars. A non-linear regression plot comparing the different gas concentrations to the mean number of progeny after six weeks was drawn. Curve 2D software version 5.01. Systat software, Inc. San Jose, California, USA and

SigmaPlot, version 12.5. Systat Software, Inc., San Jose California, USA were used for plotting the regression plots.

The second experiment assessed the role exposure times play in the control of strongly resistant populations of *R. dominica*. Target concentrations of 150 ppm and 300 ppm were categorized as 'low dose' and 'high dose' and were selected based on my first experiment, and the exposure times were 2, 4, 6, 8 and 10 days. The measured concentrations in the jar for the "low dose" averaged 232.71±15.90 while the concentrations for the 'high dose' averaged 481.44±36.15. Number of adult insects that emerged weekly for six weeks were counted in both treated and untreated jars, and the mean number of progeny after six weeks was calculated. Progeny counts were subjected to three-way analysis of variance (ANOVA) to determine significant differences ($P < 0.05$) of main effects (concentration, resistant population location and days) and their interactions (SAS Institute, 2012). One-way ANOVA was used to compare significant differences between the fumigation exposure days ($P < 0.05$) and means separated by Ryan-Einot-Gabriel-Welsch multiple range test (REGWQ) (SAS Institute, 2012). SigmaPlot, version 12.5. Systat Software, Inc., San Jose California, USA was used for plotting the bar graphs.

## Results

Table 3-1 shows the Probit analyses from the dose response assays of adult *R. dominica* to characterize the level of resistance for the two strongly resistant populations, Belle Glade, FL and Minneapolis, KS. Concentrations of phosphine required to kill 50 and 99% of the laboratory-susceptible *R. dominica* strain were compared with these two populations. The resistance ratio factors, $RR_{50}$, were subsequently calculated using the estimated $LC_{50}$ values. The $RR_{50}$ values for Belle Glade, FL and Minneapolis, KS were 927- and 761-fold, respectively, compared with the susceptible laboratory strain.

The effect of phosphine concentrations of 200 ppm and 1000 ppm applied for 48 hours on the mixed life stage colonies of six *R. dominica* populations is presented in Tables 3-2 and 3-3. The mean number of treated emerged progeny was significantly lower in both concentrations ($P<0.05$). The 200-ppm treatment completely suppressed colonies of the susceptible strains, USDA and Starbuck, and the two weak resistant strains, Junction City-2 and Wamego. Progeny were generated from cultures of Belle Glade and Minneapolis treated with 200 ppm phosphine, although the mean emergence for both was significantly different than emergence from the untreated controls (Table 3-2). All populations of LGB but Belle Glade had their progeny completely killed at a target phosphine concentration of 1000 ppm for a 48-h fumigation exposure time (Table 3-3).

The effect of the targeted phosphine concentrations 150, 300, 450, 600, 750, 900, 1050 and 1200 ppm during a 96-h fumigation on the emergence of progeny on Belle Glade and Minneapolis populations is shown in Figure 3-1. The regression plot shows a strong relationship between concentration and progeny emergence in these strongly resistant populations. As the target concentrations increased, the number of progeny significantly reduced. Target

concentrations above approximately 770 ppm resulted in a zero emergence of progeny in the Belle Glade, FL population while concentrations greater than 400 ppm killed all beetles in the Minneapolis KS population.

The effect of two phosphine doses on the mixed life colonies of two strongly resistant populations of Belle glade and Minneapolis after fumigation periods of 2, 4, 6, 8 and 10 days is summarized in Figure 3-2. As the length of exposure increased the number of progeny was significantly reduced in both populations. In all cases the treated mixed life stages of the two populations were significantly reduced as the exposure days increased compared to the control. At the 'lose dose" the number of emerged progeny was higher compared to the 'high dose'. The Belle Glade population had higher numbers of emerging progeny at all exposure days compared to Minneapolis at the 'low dose'. Applying phosphine concentrations at the 'high dose' (actual concentration from the jar of ≥ approx. 550 up to 700 ppm) for 6 days and over reduced all progeny in the Belle glade population. However, in the Minneapolis population the high dose (actual concentration from the jar of ≥ approx. 453 up to 700ppm) significantly reduced all progenies in 4 days and over.

## Discussion

The research reported above shows that *R. dominica* from two strongly resistant populations can be effectively controlled with phosphine by either increasing its concentration or by increasing the exposure period (Figures 3-1 & 3-2). This holds true if phosphine concentrations range between 400 and 800 ppm for 96 hours during fumigation. However, fumigations of 4 days or longer with phosphine concentrations between 450-700 ppm can control strongly phosphine-resistant populations lesser grain populations like that from Belle Glade in this study. These findings follow findings in several studies in the past. For example, at approximately 578 ppm Mills et al (1986) reported 99.2% mortality of immature stages in 6 days. In our study 100% complete kill for all progeny was achieved when the phosphine concentration was at 400 - 800 ppm over 4 days, and the same was achieved in 6 days and beyond when the concentrations were 450-700 ppm. Studies such as ones conducted by Sayaboc et al (1998) and Jin et al (1999) who tested adult *R. dominica* from the Philippines and China, respectively, produced similar results. The former reported that at approximately 714 and 506 ppm adults of the resistant *R. dominica* strain incurred 98.3% mortality in 3 days (Jin et al 1999) and 99.1% mortality in 7 days (Sayaboc et al 1998).

In the previous chapter, the determination of weak and strong resistance phenotype was based on a 20-h exposure times at 25°C for mortality at different gas concentrations. The estimated $LC_{99}$ for the resistant *R. dominica* from Belle Glade, FL was calculated to have an upper fiducial limit of 28,174 ppm during the 20-hour dose response assay. This dose is unrealistic and could never be achieved in the laboratory or the field using current technology (Afful et al 2017). Here I have revised the estimated level of resistance for Belle Glade beetles based on a 48 hours dose response assay with adults to ascertain what concentrations of

phosphine will be may be needed to completely kill all resistant *R. dominica*. The current study shows that a 48 hours dose response assay is enough to predict the levels of resistance in a population such as Belle Glade. The estimated $LC_{99}$ for this population was calculated to have an upper fiducial limit of 921 ppm which could be generated in the field with current technology such as with cylinder formulations of phosphine (Phillips et al. 2012). In a similar study carried out by Opit et al (2012) it was determined that $LC_{99}$ values for the resistant *R. dominica* population using a 72 hours fumigation was 3,430 ppm. This high concentration of gas will be difficult to generate under current protocol and the $LC_{99}$ in this study can be said to be reasonable and easily generated. Lorini et al (2007), like Opit et al (2012), reported levels of resistance in populations of Brazilian *R. dominica* using a 48 hours dose response and achieved a higher concentration as well (7190ppm, upper fiducial limit) of phosphine ($LC_{99}$). My 48 hr results reported here also have different $RR_{50}$ values compare to those reported for the 20 h exposures of adults in Chapter 2. Here the resistance ratios are several 100 points higher than those reported in Chapter 2, and they are more closely separated, with Belle Glade having a 1.2-fold higher ratio here compared to a 5.9-fold different in Chapter 1. The results for the 48 h assays confirms both populations have strong resistance phenotypes, and that they are more similar in the expression of this resistance than the data from the 20-h studies suggested.

The work in Chapter 2 identified weak resistant phenotypes, and one objective in that study was to make recommendations on how to control such populations. The present study found that mixed life stages of the three weakly resistant populations studied could be killed in 48 hours using concentrations of 260-920 ppm based on our target concentrations of 200-1000 ppm. These findings support those of Nayak et al (2015) who predicted that weak resistance phenotype may require phosphine concentrations of 10-fold to 50-fold greater than those needed

to kill susceptible beetles, and beetles with strong resistance that may require 100-fold or greater concentrations relative to susceptible beetles. However, the target concentration of 200 ppm could not completely control the strong resistance populations of Belle Glade and Minneapolis in 48 hours.

Kaur and Nayak (2015) sought to investigate the interaction of a range of phosphine concentrations and fumigation temperatures against strongly resistant *C. ferrugineus* found that phosphine concentration and temperature both contributed significantly to the lethal time which all the resistant individuals of a population will be eliminated with concentration being the dominant variable ($LT_{99.9}$). The study confirmed that concentration plays a similar role as was found in my dissertation, but temperature was not varied in my experiments. The study found that across all concentrations, $LT_{99.9}$ of the strongly resistant *C. ferrugineus* population was longest at the lowest temperature and shortest at the highest temperature. According to Kaur and Nayak (2015) to eliminate a strongly resistant *C. ferrugineus* 1.0 mg/L (719ppm) of phosphine is required for 20, 15 and 15 days, 1.5 mg $L^{-1}$ (1078ppm) for 12, 11 and 9 days and 2.0 mg $L^{-1}$ (1438 ppm) for 10, 7 and 6 days at temperatures of 25, 30 and 35 °C, respectively. Similarly, fumigation periods of 5 and 7 days were required for 1.0 mg/L (719 ppm) of phosphine at 25°C to attain population extinction of mixed-age populations of strongly resistant *R. dominica* from Australia and India (Collins et al. 2005, Rajendran and Gunasekaran 2002). My fumigations were done at 25°C and the amount of phosphine concentration needed to completely eradicate strongly resistant individuals in 10 days ranged between 730-870 ppm which is half less than was required in the case of *C. ferrugineu*s and almost similar to what was required to completely kill all strong resistant *R. dominica* in India and Australia.

The approved label rate of phosphine in the United States ranges from a minimum of 200 ppm up to 3625 ppm/1000 cu ft under temperature conditions of $25^0$C and minimum fumigation period of 48 hours for non-fresh commodities (US EPA 2014). From my study the concentrations I generated fall in line with these recommended rates making phosphine still usable as a fumigant in the United States to manage resistant *R. dominica*. This study looked at these control strategies in the lab and thus will recommend these phosphine doses for use in the field to ascertain the effectiveness of these.

In conclusion, protocols developed in the present study can provide the grain industry with some flexibility in application of phosphine at a range of concentrations and exposure times for management of infestations of strongly resistant *R. dominica*. This type of flexibility allows grain managers to operate more effectively and economically, provided they observe good fumigation practices such as proper sealing of bins and monitoring of gas during fumigation. Given the findings from this study I conclude that phosphine can be sustained for use into the foreseeable future.

# References

**Afful E., Elliott B., Nayak M.K. and T. W. Phillips 2017.** Phosphine Resistance in North American Field Populations of the Lesser Grain Borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). J Econ Entomol.

**Ahmedani M.S., Khaliq A., Aslam M., Naz S. and M. Y. Ahmedani. 2006.** Fumigation technique used in house type godowns for the management of resistant *Trogoderma granarium* larvae. Pak. J. Arid Agric., 9: 5-11.

**Athie I. A. and K.A. Mills. 2005**. Resistance to phosphine in stored-grain insect pests in Brazil. Braz. J. Food Technol., 8: 143-147 (2005).

**Benhalima H., Chaudhry M., Mills K. and N. Price 2004**. Phosphine resistance in stored-product insects collected from various grain storage facilities in Morocco. J Stored Prod Res 241-249.

**Bell C.H. 1979.** The efficiency of phosphine against diapausing larvae of *Ephestia elutella* (Lepidoptera) over a wide range of concentrations and exposure times. J Stored Prod Res 15 (2):53-58.

**Borah B, and Chahal B 1979.** Development of resistance in *Trogoderma granarium* Everts to phosphine in the Punjab. Plant protection bulletin.

**Bui C.H. (1998).** Some initial results on phosphine resistance of major product insect pests in Vietnam. In: Jin Z, Liang Q, Tan X, Guan L (eds.), Stored product protection. Proceedings of the 7th International Working Conference on Stored-product Protection, Beijing, China. Chengdu: Sichuan Pub. House of Sci. Technol., pp: 648-652.

**Cato A., Elliott B., Nayak M.K. and T.W. Phillips 2017.** Geographic Variation in Phosphine Resistance Among North American Populations of the Red Flour Beetle (Coleoptera: Tenebrionidae). J. Econ. Entomol 1359-1365.

**Champ B.R, Dyte C.E. 1976.** Report of the FAO global survey of pesticide susceptibility of stored grain pests FAO.

**Chaudhry M. 2000.** Phosphine resistance. Pesticide Outlook 11(3):88-91.

**Chittenden F.H. 1911**.The lesser grain borer and the larger grain borer Bulletin of United State Bureau of Entomology, 96**:**29-47 **(**1911).

**Collins P.J., Daglish G.J., Bengston M., Lambkin T.M and H. Pavic 2002.** Genetics of resistance to phosphine in *Rhyzopertha dominica* (Coleoptera: Bostrichidae). J. Econ. Entomol 862-869.

**Collins P.J. 1998.** Resistance to grain protectants and fumigants in insect pests of stored products in Australia. In: Banks HJM Wright EJ, Damcevski KA. [ed.,] Stored grain in Australia Canberra, CSIRO Stored Grain Res. Lab., pp: 55-57.

**Collins P.J., Daglish G.J., Pavic H. and R.A. Kopittke. 2005.** Response of mixed-age cultures of phosphine-resistant and susceptible strains of lesser grain borer, *Rhyzopertha dominica*, to phosphine at a range of concentrations and exposure periods. J Stored Prod Res 373-385.

**Daglish G.J., Collins P.J., Pavic H. and R.A. Kopittke. 2002.** Effects of time and concentration on mortality of phosphine-resistant *Sitophilus oryzae* (L) fumigated with phosphine. Pest Manag Sci. 1015-1021.

**Emery R.N., Nayak M.K. and J.C. Holloway. 2011.** Lessons learned from phosphine resistance monitoring in Australia. Stewart Postharvest Rev. 1-8.

**Edde P.A. 2012**. A review of the biology and control of *Rhyzopertha dominica* (F.) the lesser grain borer.J Stored Prod Res. 1-8

**Ho S. and R. Winks 1995**. The response of *Liposcelis bostrychophila* Badonnel and *L. entomophila* (Enderlein)(Psocoptera) to phosphine.J Stored Prod Res. 191-197.

**Jagadeesan R., Collins P.J, Daglish G.J., Ebert P.R. and D.I. Schlipalius 2012.** Phosphine resistance in the rust red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae): inheritance, gene interactions and fitness costs. *PLos* one **7** (2): e31582.

**Jin Z, Liang Q, Liang Y, Tan X, Guan L 1999.** Stored Products Protection. Proceedings of the Seventh International Working Conference on Stored-product Protection. 14–19 October 1998, Beijing, China, Sichuan Publishing House of Science and Technology, Chengdu, People's Republic of China pp 607-611.

**Kaur R, Subbarayalu M, Jagadeesan R, Daglish G, Nayak M, Naik H, et al 2015.** Phosphine resistance in India is characterized by a dihydrolipoamide dehydrogenase variant that is otherwise unobserved in eukaryotes. Heredity 115 (3):188.

**Lorini I., Collins P.J., Daglish G.J., Nayak M.K. and H. Pavic 2007.** Detection and characterisation of strong resistance to phosphine in Brazilian *Rhyzopertha dominica (*F.)(Coleoptera: Bostrychidae). Pest Manag Sci 63 (4):358-364.

**Manivannan S. 2015.** Toxicity of phosphine on the developmental stages of rust-red flour beetle, Tribolium castaneum Herbst over a range of concentrations and exposures. J. Food Sci. Technol 6810-6815.

**Mau Y.S., Collins P.J., Daglish G.J., Nayak M.K. and P.R Ebert.** The rph2 gene is responsible for high level resistance to phosphine in independent field strains of *Rhyzopertha dominica*. *PloS* one **7**(3): e34027.

**Mau Y.S., Collins P.J., Daglish G.J., Nayak M.K. and P.R Ebert.** The rph1 gene is a common contributor to the evolution of phosphine resistance in independent field isolates of *Rhyzopertha dominica*. *Plos* One ;7(2): e31541.

**Mills, K.A. 1986.** Phosphine dosages for the control of resistant strains of insects. In Proceedings of the GASGA Seminar on Fumigation Technology in Developing Countries.

**Nayak M.K., Daglish G.J. and T.W. Phillips 2015.** Managing resistance to chemical treatments in stored products pests. Stewart Postharvest Rev. 11(1).

**Opit G, Phillips T.W., Aikins M. J .and M. Hasan 2012.** Phosphine resistance in *Tribolium castaneum* and *Rhyzopertha dominica* from stored wheat in Oklahoma. J Econ Entomol. 1107-1114.

**Phillips T. W., Thoms E. M., DeMark J. and S. Walse 2012**. Chapter 14. Fumigation, In Hagstrum D. W. Phillips T. W. Cuperus G. (Eds.), Stored product protection. Kansas State University. Manhattan, KS. 157– 177.

**Rajendran S and N. Gunasekaran. 2002.** The response of phosphine-resistant lesser grain borer *Rhyzopertha dominica* and rice weevil *Sitophilus oryzae* in mixed-age cultures to varying concentrations of phosphine. Pest Manag. Sci. 277–281

**R. Davis. 2003.** in Encyclopedia of Food Sciences and Nutrition Second Ed.

**Sayaboc P., Gibe A and F. Caliboso. 1998.** Resistance of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) to phosphine in the Philippines. Philipp Entomol 12:91-95.

**Schlipalius D.I., Cheng Q., Reilly P.E., Collins P.J. and P.R. Ebert. 2002.** Genetic linkage analysis of the lesser grain borer Rhyzopertha dominica identifies two loci that confer high-level resistance to the fumigant phosphine. *Genetics* 773-782.

**Tyler, P. S., R.W.D. Taylor, and D. P. Rees** Insect resistance to phosphine fumigation in food warehouses in Bangladesh. Int. Pest Control 25: 10–13 (1983).

**United States Environmental Protection Agency. 2014.** Notification to revise "area" to "structure" and other minor text EPA Registration No. 68387-8 https://www3.epa.gov/pesticides/chem_search/ppls/068387-00008-20140313.pdf [21 August 2018].

**Valmas N and P. R. Ebert. 2006** Comparative toxicity of fumigants and a phosphine synergist using a novel containment chamber for the safe generation of concentrated phosphine gas. PLoS ONE. PLoS ONE **1**(1): e130. doi:10.1371/j. pone.0000130.

**Winks R. 1984.** The toxicity of phosphine to adults of *Tribolium castaneum* (Herbst): time as a dosage factor. J Stored Prod Res 45-56.

**Yan X.P., Li W.W., Liu Z.W., Qin Z.G., Wu X.Q., Song Y.C. and Z.P. Shen. 2004.** Investigation of phosphine-resistance in major stored grain insects in China. Grain storage (in Chinese with English abstract). 32: 17-2.

**Table 3- 1 Probit analyses to estimate lethal concentrations from dose-mortality studies with adult *Rhyzopertha dominica* from a lab susceptible strain and two selected strong-resistant populations with 48 hr exposure to gas at 25 °C**

| Population | $LC_{50}$ (95% FL) ppm | $LC_{99}$ (95% FL) ppm | Slope | Intercept | $RR_{50}$ | $\chi^2$ (df) | P-value |
|---|---|---|---|---|---|---|---|
| Minneapolis | 495.16 (363.48-552.54) | 732.84 (696.64-813.28) | 1.86 | -10.53 | 761.78 | 35.3 (19) | 0.96 |
| Belle Glade | 602.57 (570.72-625.72) | 870.12 (836.55-921.18) | 6.33 | -40.53 | 927.03 | 16.02 (13) | 0.25 |
| USDA | 0.65 (0.62-0.69) | 1.32 (1.15-1.66) | 7.61 | 1.40 | 1.00 | 7.04(4) | 0.13 |

$LC_{50}$ and $LC_{99}$ are the lethal concentrations of phosphine exposed for 48 h estimated to kill either 50% or 99% of a given population. $RR_{50}$ refers to the resistance ratio as calculated from the $LC_{50}$ for a given population divided by the LC50 for the USDA susceptible strain.

**Table 3-1. Mean adult progeny emerged from mixed life stage cultures 6 weeks after treatment with 200 ppm of $PH_3$ for 48 h compared to untreated control.**

| Population | Mean Emerged Treated ± SE | Mean emerged Control ± SE |
|---|---|---|
| USDA | 0 | 715.67± 6.88 |
| Starbuck | 0 | 506.67± 8.67 |
| Junction City-2 | 0 | 492.67±12.99 |
| Wamego | 0 | 480.33±0.33 |
| Minneapolis | 263.67±8.08 | 430.67± 4.48 |
| Belle Glade | 296.67±11.01 | 489.33± 4.70 |

**Table 3-2. Mean adult progeny emerged from mixed life stage cultures 6 weeks after treatment with 1000ppm of $PH_3$ for 48 h compared to untreated control.**

| Population | Mean Emerged Treated ± SE | Mean emerged Control ± SE |
|---|---|---|
| USDA | 0.00 | 628.67 ± 15.59 |
| Starbuck | 0.00 | 409.67 ± 5.36 |
| Junction City-2 | 0.00 | 433.33 ± 15.62 |
| Wamego | 0.00 | 377.00 ± 9.71 |
| Minneapolis | 0.00 | 352.33 ± 6.06 |
| Belle Glade | 143.00 ± 15.58 | 388.67 ± 9.24 |

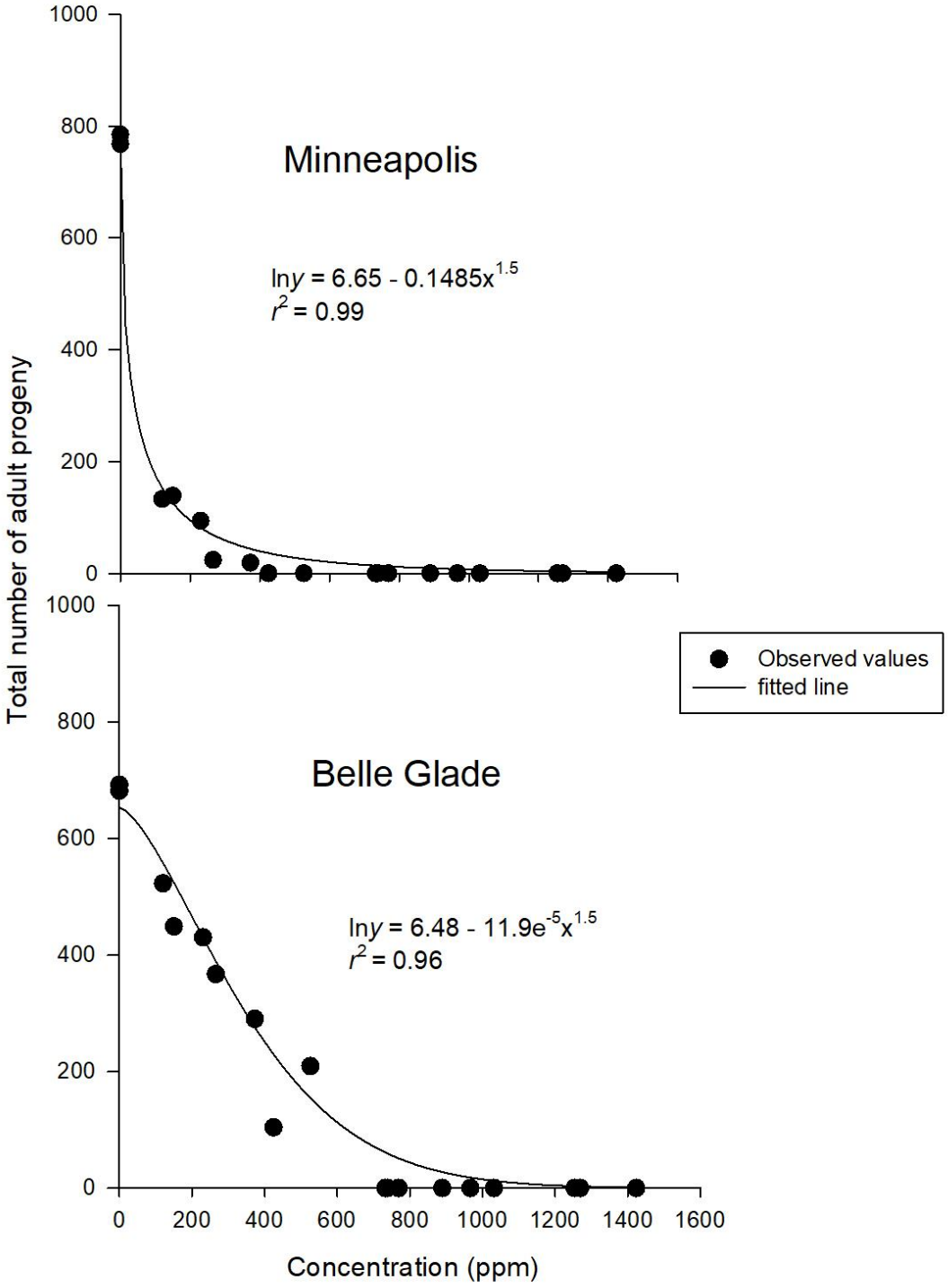


**Figure 3-1. Effect of phosphine concentrations held for 96 h on the number Belle Glade and Minneapolis progeny emergence six weeks after fumigation**

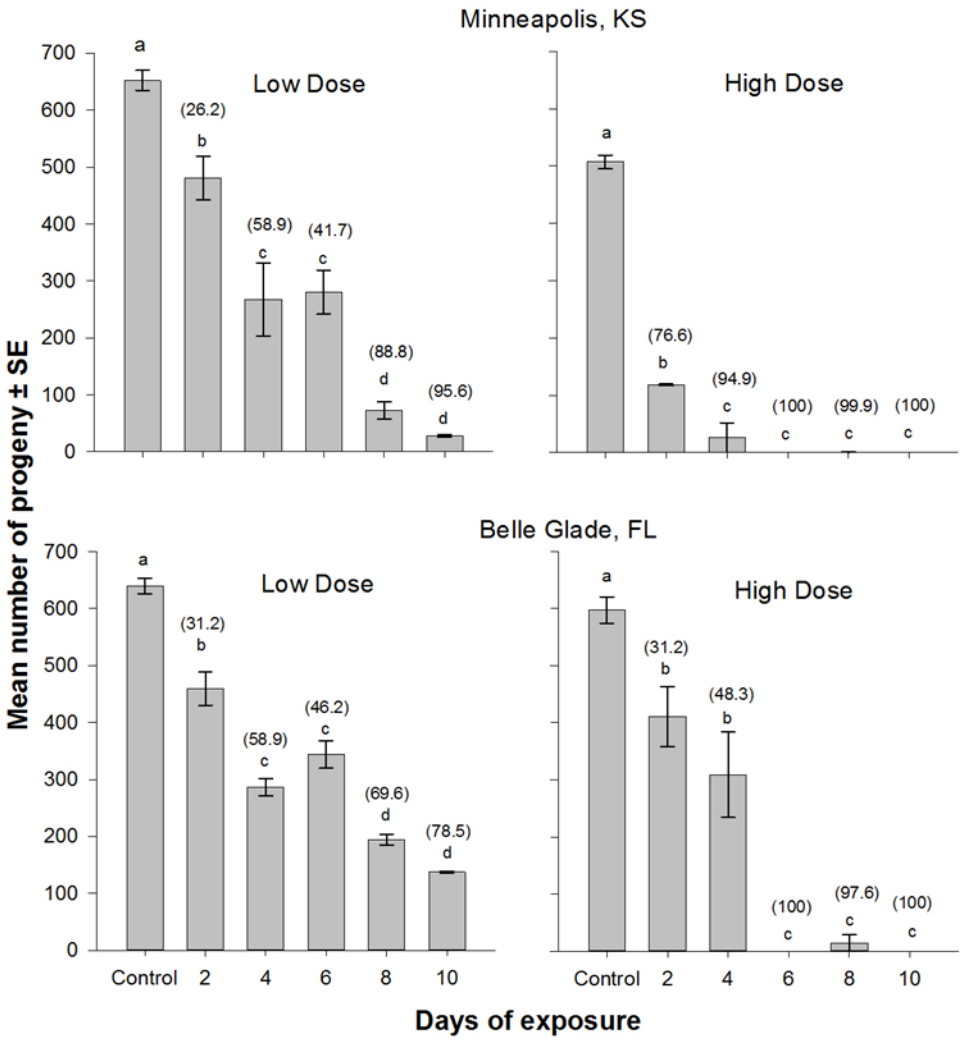


**Figure 3-2. Mean ± SE number of adult progeny of *Rhyzopertha dominica* produced at 6 weeks after a 2,4,6, 8 and 10 d exposure to target phosphine concentrations of 150 (low) and 300ppm (high). Number above each error bar is percentage reduction of adult progeny production after exposure to treatment relative to production on untreated individuals. Bars with different letters are significantly different (F, range = 64.31 – 331.24; df = 5, 9; P < 0.0001; one-way ANOVA with means separated by REGWQ multiple range test.**

# Chapter 4 - Utility of a Knockdown Assay to Determine Phosphine Resistance in *Rhyzopertha dominica* (Coleoptera: Bostrichidae), the Lesser Grain Borer

## Abstract

Insect resistance to phosphine is a global problem of which the United States is no exception. Detection of resistance relies on the so-called FAO bioassay which relies on a 20-hour fumigation and 14-day post fumigation assessment. This laborious and technical process is impractical for early detection and monitoring of phosphine resistance. Phosphine applied at high concentrations have been found to elicit a knockdown effect of either susceptible or resistant insects. Using several populations from chapter 2 of this dissertation, the relationship between adult knockdown time and the FAO method utilizing knockdown for single beetles, 50% and 100% knockdown time for groups of beetles was assessed. Using a phosphine concentration of 3000 ppm this study sought to determine the most effective knockdown time among knock down times at 100%, 50% and single insects. Also, the most effective concentrations among 5 that will elicit the quickest knockdown while estimating the recovery times from these set of concentrations. Results from the study indicates that $KT_{100}$ quick test was better than the $KT_{50}$ and $KT_{single}$, the $KT_{50}$ and was determined to be more efficient because bioassays were able to clearly distinguish among susceptible, weak and strong resistance. A $KT_{100}$ of 18 minutes approximately based on only susceptible populations from the study will be a potential viable knock time to distinguish a susceptible population from a resistant. The potential of recovery time as a new technique in determining resistance showed that higher concentrations of phosphine significantly elicited a quicker recovery in highly resistant populations of *R dominica* compared to a susceptible population. These findings have potential of establishing a robust commercial kit for phosphine resistance detection and could be incorporated in a resistance management program.

# Introduction

Storage cereal grains and legumes requires that infestation by insects and other pests is effectively prevented or controlled (Subramanyam and Hagstrum 2012). The lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae) is one of most severe pest of stored wheat and rice worldwide. The damage of the pest is distinctive and heavy as both adults and larvae feed on germ and endosperm of the target grain reducing kernels to powder (Gundu Rao and Wilbur 1972). Several methods of control have been employed to control the lesser grain borer, with fumigation being the most effective method (Edde, 2012). The fumigant hydrogen phosphide ($PH_3$), commonly referred to as phosphine, is a widely used toxin that have been in use for over half a century due to its ideal properties. The solid formulations of this toxin are easy to apply relatively inexpensive compared to other fumigants, it is effective against invertebrate and vertebrate pest species and it does not leave toxic residues on treated commodities making it an important tool in the management of insects associated with stored grains (Chaudhry, 2000). However, these ideal qualities of phosphine have led to an increased dependence on the fumigant. The constant and long-term use of a single pesticide is likely to increase the risk of resistance evolving and becoming established.

A global survey was undertaken during 1972-1973 by the United Nations' Food and Agriculture Organization (FAO) to assess the presence or absence of insects resistant to phosphine by using a discriminating dose bioassay. The results of the survey showed that about 10% of the collected populations contained phosphine resistant individuals (Champ and Dyte, 1976). After this FAO survey there were reports of wide-spread cases of phosphine resistance in several species of stored-product insects in many countries. In the early 2000s it was reported

that phosphine resistance was prevalent in at least 11 species of stored-product insects in 45 countries, with the list growing (Chaudry, 2000).

In the last several years, detection of phosphine resistance in stored product insect populations relied on the so-called FAO method number 16 (Food and Agriculture Organization 1975). The FAO method stipulated that adult insects were to be exposed to a discriminatory dose of phosphine for twenty hours at 25°C. The gas was vented after the 20 hr exposure and the test insects held in clean air for 14 days to allow for recovery or delayed mortality. Insects that survived this 20-hour exposure and the 14-day post fumigation were considered resistant while those surviving were susceptible. Studies conducted in Australia have identified two resistant phenotypes for *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica*, the strong and weak phenotypes (Collins et al 2005, Schlipalius et al 2002). Further genetic analysis of these insect species determined that mutations at two genetic loci conferred resistance in both *R. dominica* and *T. castaneum* (Jagadeesan et al 2012; Mau et al 2012a; Mau et al 2012b). The first gene, rph1 (tc-rph1 for *T. castaneum*), is responsible for weak resistance, whereas the second, rph2, acts synergistically with rph1 to confer strong resistance (Schlipalius et al 2002). The "weak" resistance phenotype may require phosphine concentrations of 10-fold to 50-fold greater than those needed to kill susceptible beetles, and beetles with the "strong" resistance phenotype may require 100-fold or greater concentrations for control relative to susceptible beetles (Nayak et al 2015).

The FAO method has been successful in determining the presence or absence of resistance in a given insect population. However, one limitation for using the FAO method to help with control decisions is the wait time of 14 days needed to ascertain the presence or absence of resistance. Additionally, this method is laborious and technical for use by the grain

industry as it requires key analytical instruments such as gas chromatography and methods to generate phosphine for the test. The FAO test therefore requires special training and handling which is impractical for grain managers or commercial fumigators. These demerits of the FAO method have led to interest in having a simple one-day test that is practical and inexpensive.

Several quick test" studies for phosphine resistance are based on a behavior of adult insect on exposure to high levels of phosphine known as narcosis, which is assessed by observing and recording treated adult knockdown that usually does not cause death (Bang and Telford, 1966; Winks, 1985; Reichmuth, 1991, 1992; Wontner-Smith et al., 1999). When normal susceptible insects are exposed to phosphine at a high concentration (around 0.4 mg/l, 300 ppm, or higher) they enter a narcotized state wherein most of the insects will become inactive and be active again if phosphine is made unavailable (Reichmuth, 1991). Based on this behavior, resistant insects are expected to tolerate longer times under gas before knockdown, and susceptible insects should be knocked down at shorter times.

A lot of elements are required to develop a robust rapid assay. Studies have identified the amount of phosphine concentrations, the time to knockdown, susceptibility or otherwise of the insect population and what defines a knockdown or narcosis (Reichmuth, 1991, Bell et al 1994; Cao and Wang 2000; Winks, 1985,1994; Waterford and Winks, 1994). Commercially, the only quick test designed for use by grain fumigators is that formulated based on a study by Steuerwald et al. (2006) (Degesch Inc. http://www.degeschamerica.com). This study looked at the time to knockdown within 30 minutes of adult insects for the most common stored-product pest insects exposed to a phosphine concentration of 3000 ppm. The Resistance-Test-Kit from the study consisted of a 100 ml syringe, a 5-L flexible plastic canister, a cannula with a rubber hose and special magnesium phosphide pellets (2) that was able to generate about 4000-6000 ppm of

phosphine gas. This study did not investigate narcosis associated with an important pest of stored grain; the lesser grain borer.

In the chapter 2 of this thesis I utilized the FAO assay to determine the levels of resistance in 34 geographical locations in North America (Afful et al. 2017). Thirty- two out of the 34 locations sampled reported varying levels of phosphine resistance indicating a big problem in North-America. Using several populations (18) from this study the general objective of the study was to assess and improve the ability of a commercial phosphine resistance quick test to produce results as reliably as the commonly used discriminating dose bioassay. Specifically,

1. Assess the concentrations of phosphine needed to elicit the quickest knock down times using adult *R. dominica* from populations previously classified as susceptible, weakly and strongly resistant.
2. Study the effect of a sustained physical stimulus on knockdown times for adult beetles.
3. Determine the most efficient knockdown time technique in single, or groups (50% and 100%) of adult *R. dominica* populations after comparison to the well-known FAO method.
4. Evaluate the relationship between knockdown time and recovery time at a given concentration of *R. dominica* populations classified as susceptible, weakly and strongly resistant.

# Materials and methods

## *Rhyzopertha dominica* rearing

Insect rearing techniques followed similar ones employed by Afful et al 2017. Twenty *R. dominica* populations from that paper were used for this study with three: USDA, Wamego and Belle Glade that had been categorized as susceptible weak and strong resistant, respectively, and were selected for some extra analysis. Stock cultures of insects used originated from field-collections and had been propagated in the lab for two years under standard conditions (Afful et al 2017). The lab susceptible reference strain, referred to as USDA, originated from a reference population maintained at the United States Department of Agriculture (USDA), Center for Grain and Animal Health Research, Manhattan, Kansas, for over 40 years. Adult beetles from each location were accumulated in 473-ml wide mouth Mason jars (Hearthmark, LLC, Fishers, IN), closed with a ring-lid equipped with a metal screen and filter paper to prevent the adults from escaping the jar but allow air and moisture exchange. Beetles were held on a mixture of 95% whole wheat kernels and 5% wheat flour, in a growth chamber kept at constant regimes of 28°C, 65% relative humidity (RH) and a photoperiod of 16:8 (L:D) h until used in the quick test assay.

## Phosphine concentration generation and exposure chamber

In this study I used a PYREX® 55mL Screw Cap Culture Tubes with PTFE Lined Phenolic Caps as a fumigation chamber that was intended to mimic the 100 ml syringe exposure chamber of the Degesch test kit (Steuerwald et al. 2006). The glass tube measured 25x150 mm and a gas-tight Fisherbrand™ Turnover Septum Stopper served as the inlet to introduce phosphine gas. To assess the effect of phosphine concentrations on knockdown times, two experiments were performed. In the first experiment we tried to imitate the methods employed

by Steuerwald et al. (2006) for which a phosphine concentration of 3000 ppm was used in testing the time to knockdown of *R. dominica* from our previous study. A Hamilton® 25 mL, Model 1025 TLL gas tight Syringe were used to transfer 16.5 mL of 1% (10,000ppm) phosphine to fumigation chambers from gas tight CEL Scientific Tedlar® PVF film bags containing previously cylinderized phosphine. This volume of 1% phosphine was determined to bring the fumigation chambers to approximately 3000 ppm. In the second experiment we assessed the use of 5 different exposure concentrations (500,1000, 2000, 3000 and 5000 ppm) on the knockdown times using adults from three *R. dominica* populations established in Chapter 2 as having phenotypes of phosphine susceptible, both weak resistant and strong resistant from our previous study. The volumes needed for the fumigation jars for each concentration were 2.75 ml, 5.5 ml, 11 ml, 16.5 ml and 27.5 ml and these brought the phosphine concentrations to the desired targets, respectively. Analysis of the fumigation chambers once gas was added was done by a GC-FPD as described below.

**Quantitative GC Analysis**

Phosphine concentration analysis in each fumigation chamber was done according to methods in Cato et al 2017 and Afful et al 2017 using quantitative gas chromatography (GC) gas in to a Shimadzu GC-17A (Shimadzu, Kyoto, Japan) equipped with a GS-Q column (30 m long × 0.53 mm i.d., 0.25 μm film thickness, J & W Scientific, Folsom, CA) and a flame photometric detector set in the phosphorous mode. All gas samples from the fumigation chambers were injected onto the GC with a Hamilton® 25 μL, Model 1702 N SYR, Cemented NDL, 22 ga, 2 in, gas tight syringe. The operating conditions of the GC are as follows: injector temperature of 200°C, detector temperature of 200°C, column flow rate of 4 mL/min, and oven temperature of

150°C in split injection mode. Ultra high purity helium purchased from Linweld (Lincoln, NE) was used as the carrier gas. A 200-ppm phosphine standard was achieved by the dilution of 1% phosphine mixed into a carefully measured volume of air in a CEL Scientific Tedlar PVF film bag. Injections of this standard gas at volumes of 25, 20, 15, 10, and 5 µl were analyzed. The 15-µl injection is in this case equivalent to the standard of 200ppm

## Comparison between FAO method and different knockdown time techniques

In this study I utilized the two main techniques of developing a rapid assay: the time to knockdown of 50% ($KT_{50}$) and the time to knockdown of 100% ($KT_{100}$) of adult *R. dominica* for any given population. I also investigated the time to knockdown of single individuals from a population mainly for comparison to test the efficiency of this test compared to the well-established methods of knockdown assays. Adults from eighteen *R. dominica* populations studied in Chapter 2 with known FAO frequencies were assessed using these three methods. Each technique was assessed using a phosphine concentration of 3000 ppm as reported by Steuerwald et al. (2006). In testing for $KT_{50}$ and $KT_{100}$, 10 insects from each population were used with 5 replications. In this study the definition of knockdown time is that utilized by Cato 2015, who explained knockdown time as the time at which there is a persistent and complete lack of movement by a treated insect. Time to knockdown for each of my assays was recorded when there was a complete lack of movement for at least thirty seconds after insects exposed to phosphine for either 50% or 100% of the group of beetles in a fumigation tube. In the single trial experiment, we used one insect per replication (n=10). All the tested single insect trials, $KT_{50}$, and $KT_{100}$ were compared with the known FAO frequencies using a linear regression and one-way ANOVA was used to show if there were differences between knock down time techniques used, between locations of the *R. dominica* strains ($P < 0.05$). Means were separated by Ryan-

Einot-Gabriel-Welsch multiple range test (REGWQ) (SAS Institute, 2012). Graphs were plotted using SigmaPlot, version 12.5.

**Knockdown and Recovery Time Assay**

Using three categorized *R. dominica* populations based on a dose response assay in chapter 2 which included susceptible (USDA), weak (Wamego) and strong resistant (Belle Glade) I assessed the relationship between knockdown time 100% and recovery time. Recovery time has been defined as the time that all previously knocked down adult beetles returned to persistent moving of all its legs. This observation of recovery was recorded for each individual insect in the group until the last one moved for a minute. When all in the group move at a given time that is recorded as the recovery time for that replicate of the given population. Each population tested had 10 insects replicated five times. Five phosphine concentrations 500, 1000, 2000, 3000 and 5000 ppm were first tested for $KT_{100}$ and after carefully and quickly ventilating the tube the time to knockdown was started. Two-way ANOVA with Ryan-Einot-Gabriel-Welsch multiple range test (REGWQ) were used to identify significant variation between recovery time and knockdown time.

**Effect of stimulus during knockdown trials**

Preliminary studies suggested evaluation of beetles stimulated by other beetles in a group being assayed had longer time to knockdown then would single *R. dominica* alone under phosphine. Following methods by Cato (2015), this effect was tested for two populations from Chapter 2. A resistant *R. dominica* population Junction city 1 with a resistance frequency of 57% and a USDA susceptible population was used for this assay. I used single insects from the two

populations each receiving set of treatment we termed with stimulus and without stimulus. Stimulus treatment involved phosphine treated vials held on the bench horizontally with a single beetle per population being rolled a complete 360° in one direction and then 360° back to the starting position at one-minute intervals until a 30-sec knock down was achieved. A non-stimulus treatment involved not moving the vials at all during the full duration of the knockdown experiment. Each population, susceptible (n=40) and the resistant (n=40), were ran with stimulus and without stimulus. These two populations were then compared independently using an Unpaired T-Test.

## Results

Table 4-1 shows results for the three techniques used in predicting knockdown times from 18 populations that have been assessed for frequencies of phosphine resistance using the FAO method from Afful et al 2017. The single beetle technique, $KT_{single}$ had average knockdown times ranging from 6.01- 59.52 minutes among the populations. The knockdown test using $KT_{50}$ had average times ranging from 5.91- 41.31 minutes while $KT_{100}$ technique had times that ranged from 10.41- >300 minutes. Using ANOVA, the variation between the quick test treatments among *R. dominica* populations were also determined. Generally, there were significant differences among the three tests for each population and among all populations with each the tests separately ($p<0.01$). Further post hoc analyses revealed that among all three techniques the $KT_{100}$ was statistically different from the other two tests for all 18 populations assayed. Eight of the 18 populations had $KT_{single}$ and $KT_{50}$ not different from each other with the other 10 knockdown times significantly different.

A similar post hoc analysis was carried to determine the differences among the populations within each knockdown technique. The single insect trials revealed that most of the populations were significantly different from each other except for Stirling and Coronach which recorded average knockdown times of 11.04 minutes. A similar observation was encountered in Princeton, Parlier 2 and Garden City with an average knockdown time of 19.51 minutes. These six populations had very similar FAO resistance frequencies. The $KT_{50}$ test had a lot of variability among the populations. The USDA lab susceptible population and Belle glade which was categorized as a strong resistant population was significantly different from all the other populations. There was not a general trend of groupings as most of the populations significantly differed from each other. The KT100 technique of assessing knockdown time showed the best

separations of populations by FAO resistance frequency. Populations that had FAO resistance frequencies from 0-4% were significantly different from the other populations with KT times between 10.41-17.37 minutes. The knockdown time of the population categorized as strong resistant Belle Glade was significantly different from the other 17 with time of over 300 minutes. The two populations Parlier 1 and Minneapolis with the highest FAO frequencies were also significantly different from the others with an average time of 193.61 minutes. Results also showed that populations that had FAO resistance frequencies between 71-93% were not significantly different from each other with knockdown times ranging from 77.19- 104.50 minutes. However, Burleson which fell in this range of frequencies was significantly different from these five with a knockdown time of 134.06 minutes.

Figure 4-2 shows a regression plot for the raw data on knockdown time collected for each of the three techniques as a function of the FAO resistant frequencies previously determined for these 18 *R. dominica* populations from Table 4-1. The ability of resistance frequency in a population to explain the time-to-knockdown according to the $KT_{single}$ was estimated with an $r^2$ value of 0.56 ($p<0.0001$) while that of $KT_{100}$ was 0.55 ($p<0.0001$). The relationship between $KT_{50}$ and FAO resistance frequencies was the least with an $r^2$ value of 0.28 ($p<0.0001$).

The role that a physical stimulus played in knockdown time for single beetles from two populations categorized as susceptible and resistant by the FAO assay is summarized in Figure 4-2. There was a significant difference between insects treated with stimuli and those untreated ($p<<0.01$ and $p<0.03$). The mean knockdown time for susceptible beetles without any stimulus applied to the assay vial was 6.05 minutes (SE=0.23) (n=40), while beetles stimulated by rolling the exposure vial took 7.03 minutes (SE=0.31) (n=40) to knockdown. A t-test analysis using the raw data showed a statistical difference indicated by a p-value<<0.01. The resistant population

mean knockdown time recorded a similar trend. While the beetles without stimulus was 14.16 (SE=1.09) (n=40) minutes, that with stimulus was 21.74 (SE=3.12) (n=40) minutes.

Figure 3 shows the effects of phosphine concentration on time to knockdown and recovery time for groups of 10 adult *Rhyzopertha dominica* from susceptible, weak and strong resistant populations. Generally, it was observed that as the amount of phosphine concentration increased the knockdown times decreased. In populations categorized as susceptible, the KT started off at 48.85 minutes at a phosphine concentration of 500 ppm decreased to 26.12 minutes at 1000 ppm, 21.12 minutes at 2000 ppm, 10.07 minutes at 3000 ppm and finally to 7.39 minutes. For those categorized as weak resistant the KT started off at 433.20 minutes at a phosphine concentration of 500 ppm decreased to 254.90 minutes at 1000 ppm, 170.02 minutes at 2000 ppm, 77.06 minutes at 3000 ppm and finally to 13.21 minutes. Lastly the resistant population had no recording of KT at the lower concentrations of 500 ppm and 1000 ppm as insects exposed to these concentrations were actively moving after 72 hours so I terminated that part of the assay. The experiments for KT began at 2000 ppm which had an average time of 495.5, a KT of 352.9 at 3000 ppm and 120.29 at 5000 ppm. A test to examine the variability between the concentrations using ANOVA showed that there were significant differences between the treatments at $p<<0.01$. Additionally, a post hoc analysis using REGWQ revealed each observed knockdown time per concentration was significantly different from each other at $P<<0.01$). The recovery times based off the knock down times is also summarized in figure 4-3. It was observed that resistant populations recovered quickly compared to susceptible and weakly resistant individuals after exposure to the different phosphine concentrations. The recovery time (RT) of the susceptible population started off at 203 minutes at 500 ppm decreased to 150.70 minutes at 1000 ppm, 107.50 at 2000 ppm, 88.02 minutes at 3000 ppm. and finally, 66.30

minutes at 5000 ppm. The weakly resistant population RTs included 251.24 minutes at 500 ppm decreased to 192.54 minutes at 1000 ppm, 168.23 at 2000 ppm, 61.26 minutes at 3000 ppm. and finally, 43.80 minutes at 5000 ppm. The shortest recovery times were recorded in the resistant population which started off at 25.62 at 2000 ppm, 15.48 at 3000 ppm and 9.90 minutes at 5000 ppm. A test to examine the variability between the concentrations using ANOVA showed that there were significant differences between the treatments at $p<<0.01$. Additionally, a post hoc analysis using REGWQ revealed each observed recovered time per concentration was significantly different from each other at $P<<0.01$).

# Discussion

The global problem of phosphine resistance has brought about the need for a better, robust and efficient way of detecting resistance without relying on the FAO assay. This study looked at three techniques of assessing knocked down times and a new way of detecting resistance: observing the recovery time. We also compared knockdown times with already established resistance frequencies of 18 geographically distinct populations based on the FAO method to obtain a much clearer understanding on how the techniques could be applied effectively. One factor that this study sought to look at was the effect an external stimulus had on knockdown times. In many rapid assay experiments insects are usually put in groups of 10-20 to assess knockdown times with limited knowledge on the role of an external stimuli plays on recording knockdown time.

My results with the $KT_{100}$ assay suggest that waiting for all insects in a group to be completely knocked down is a more efficient way of detecting resistance than others tested. The $KT_{100}$ values in Table 4-1 were all significantly greater than other values, and from a practical standpoint I found it to be an unambiguous more streamlined approach to wait for the last beetle to be knocked down a minimum of 30 seconds. The $KT_{100}$ in this study was able to properly distinguish the most resistant population from the groups categorized as susceptible or with a very low frequency of resistance. Three of the populations tested had FAO resistance frequency of 0%. The times between the three populations ranged from 10.41 minutes to 17.37 minutes which statistically were not different. Ideally more susceptible populations will be required to be able to establish the cut off times that distinguishes resistant populations from susceptible ones. However, previous studies (Opit et al 2012, Afful et al 2017) have shown that phosphine susceptibility in the field is on a high decline making these times a good baseline to predict

resistance or otherwise using our rapid assay. Steuerwald et al. (2006) established times at which insect needed to be considered knockdown to prove susceptibility. As an example, *Tribolium castaneum* population needed to be knockdown in 8 minutes or less under a phosphine concentration of 3000 ppm to be considered susceptible. In our study, based on the 3 identified susceptible populations we can predict that a knockdown time of approximately 18 minutes or less could be sufficient to categorize a given population as susceptible to phosphine, or that any population with a KD time greater than 18 min could be resistant.

My results suggest that longer KD times have utility in confidently categorizing resistance, including strong resistance, with confidence, compared to shorter times. All populations in Table 4-1 with a $KT_{100}$ at 60 minutes or greater were also found earlier to have FAO resistance frequencies of 50% or higher. The known strong resistant populations of Minneapolis and Belle Glade had $KT_{100}$ values of 211 and over 300 min, respectively. Categorization of resistance based on $KT_{100}$ is therefore a better technique compared to $KT_{50}$ and $K_{single}$. Several studies have proposed $KT_{50}$ as an efficient way of scoring resistance in a population (Cato, 2015, Cao and Wang 2000; Waterford and Winks 1994). However, my study supports $KT_{100}$ is the most efficient knockdown test, and I recommend that a commercial quick test using this metric could be developed for *R. dominica*. Other studies have proposed such a metric in the development of rapid assay (Nayak et al 2013, Steuerwald 2006, Reichmuth, 1991). Utilizing $KT_{single}$ and $KT_{50}$ will not be a better technique compared to $KT_{50}$ in estimating resistance although among the three techniques the relationship between FAO resistance and knockdown time was the highest, $r^2$=0.56. Examining the data showed that almost all the populations had knockdown times that were significantly different from each other. In both cases the population categorized as strong resistant was significant different from the other 17.

However, it was difficult to distinguish between weak and susceptible individuals from the knockdown times observed making these techniques unsuitable for a rapid assay from this study. It is true from my study that the times to knockdown are shorter in both $KT_{50}$ and $KT_{single}$ and will potentially be efficient way to estimate resistance but the fact that it is very difficult in distinguishing among the phenotypes of resistance from this study

This study sought to determine if an added physical stimulus during knockdown trials increased the time to knockdown of the beetles. We tried to simulate beetles being moved around, contacting each other, or falling over, and may be related to what they experience in groups with other beetles. Our finding has confirmed that added stimulus increases the knockdown times of a given population. Although relatively short KD times for single beetles may seem attractive for resistance screening due to the shorter time need, one drawback is that one beetle can represent only one phenotype knockdown related to phosphine resistance. Tests with 10 beetles in an exposure chamber may easily allow for observing the range of behavior in a sample due to having 10 beetles rather than one.

Determining the effective concentration that will elicit the quickest knockdown was another area my study looked at. In a study by Reichmuth (1991), adult *R. dominica* were exposed to a concentration of phosphine from 1000-3000 ppm and then checked after 30 minutes. If any insects were active after that exposure period, the population was deemed resistant (Reichmuth 1991). This range of dosage was used because Reichmuth (1991) determined that above 700 ppm, concentration was not a factor in knockdown time of the susceptible strains under consideration. This finding was contrary to what Waterford and Winks (1994) found, as they established from their study that individuals within a susceptible population showed a variation in knockdown time when exposed to 1428 ppm of phosphine. In

an earlier publication from Winks (1985), it was found that the concentration of phosphine determined the time to knockdown. In my current study I looked at the role five concentrations (500, 1000, 2000, 3000 and 5000 ppm) and realized as the concentration increased, both resistant and susceptible *R. dominica* individuals were more quickly knockdown. For strong resistant populations like Belle Glade using a concentration of about 5000 ppm is more efficient compared to using concentration of 3000 ppm as the former dose could predict resistance in 2 hours. Currently, phosphine label allows maximum dose of 3600 ppm for phosphine fumigation. Adjusting this level of dosage will go a long way in estimating phosphine resistance in the field. One critical finding in this study is the potential use of recovery time as a new tool to augment knockdown time for the design of a rapid assay. My study found that as phosphine concentration increased the recovery time was shorter for resistant populations and longer for the susceptible population. This finding will serve a confirmatory test for populations that potential are strongly resistant as my study as shown that strong resistant populations recover quicker than weak or suspectible populations

Resistance monitoring is an important tool in any IPM program and a rapid assay for the detection of resistance in *R. dominica* will provide a big boost for the grain industry. Currently there is no commercial kit designed for the detection of resistance in this important grain of stored grain and findings can be of assistance in designing such a kit.

# References

**Afful E., Elliott B., Nayak M.K. and Phillips T.W. 2017.** Phosphine Resistance in North American Field Populations of the Lesser Grain Borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae). J Econ Entomol DOI.org/10.1093/jee/tox284.

**Anonymous. 1975.** Recommended methods for the detection and measurement of resistance of agricultural pests to pesticides – Tentative method for adults of some major pest species of stored cereals, with methyl bromide and phosphine. FAO method number 16. FAO Plant Protection Bulletin 23:12-25.

**Bell, C.H., N. Savvidou, K. A. Mills, S. Bradberry, and M. L. Barlow. 1994.** A same-day test for detecting resistance to phosphine. Proceedings of the 6th International Working Conference on Stored-product Protection 1: 41-44.

**Cao, Y. and D. Wang. 2000.** Relationship between phosphine resistance and narcotic knockdown in *Tribolium castaneum* (Herbst), *Sitophilus oryzae* (L.), and *S. zeamais* (Motsch). Proc. Int. Conf. Controlled Atmosphere and Fumigation in Stored-products: 609-616.

**Champ, B. R. and C. E. Dyte. 1976.** Report of the FAO global survey of pesticide susceptibility of stored grain pests. FAO Plant Production and Protection Series, 5, FAO, Rome.

**Chaudhry M. Phosphine resistance 2000**. Pesticide Outlook 11(3):88-91.

**Collins, P. J. 1998.** Resistance to grain protectants and fumigants in insect pests of stored products in Australia. Proc. Australian Post-harvest Technical Conference: 55-57.

**Collins PJ, Daglish GJ, Bengston M, Lambkin TM, Pavic H 2002**. Genetics of resistance to phosphine in *Rhyzopertha dominica* (Coleoptera: Bostrichidae). J Econ Entomol 862-869

**Cuperus, G. W., C. K. Prickett, P. D. Bloome, and J. T. Pitts. 1986.** Insect Populations in aerated and unaerated stored wheat in Oklahoma. J of the Kansas Entomol Soc. 59(4): 620-627.

**Gundu Rao, H.R. and Wilbur, D.A. (1972)** Loss of wheat weight from feeding of lesser grain borer. J. Kansas Entomol. Soc. 45, 238–41.

**Jagadeesan R. Collins P.J, Daglish G.J., Ebert P.R., and Schlipalius D.I 2012.** Phosphine resistance in the rust red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae): inheritance, gene interactions and fitness costs. *PLos* one **7** (2): e31582

**Mau Y.S., Collins P.J., Daglish G.J., Nayak M.K. and Ebert P.R 2012.** The rph2 gene is responsible for high level resistance to phosphine in independent field strains of *Rhyzopertha dominica*. PloS one 7(3): e34027

**Mau Y.S., Collins P.J., Daglish G.J., Nayak M.K. and Ebert P.R 2012.** The rph1 gene is a common contributor to the evolution of phosphine resistance in independent field isolates of *Rhyzopertha domini*ca. Plos One ;7(2): e31541

**Nayak M.K., Daglish G.J. and Phillips T.W. 2015.** Managing resistance to chemical treatments in stored products pests. Stewart

**Opit, J. P., T. W. Phillips, M. J. Aikins, and M. M. Hasan. 2012.** Phosphine resistance in *Tribolium castaneum* and *Rhyzopertha dominica* from stored wheat in Oklahoma. J Econ Entomol 105(4): 1107-1114.

**Schlipalius D.I., Cheng Q., Reilly P.E., Collins P.J. and Ebert P.R**. **2002.** Genetic linkage analysis of the lesser grain borer Rhyzopertha dominica identifies two loci that confer high-level resistance to the fumigant phosphine. Genetics 161(2):773-782

**Reichmuth, C. 1991.** A quick test to determine phosphine resistance in stored-products research. GASGA Newsletter 15: 14-15.

**Subramanyam, B. and D. W. Hagstrum (Eds.). 2012.** Alternatives to pesticides in stored-product IPM. Springer Science & Business Media.

**Steuerwald, R., H. Dierks-Lange, and S. Schmitt. 2006**. Rapid bioassay for determining the phosphine tolerance. Proceedings of the 9th International Working Conference on Stored-Product Protection: 306-311.

**Waterford, C. J., and R. G. Winks. 1994.** Correlation between phosphine resistance and narcotic response in *Tribolium castaneum* (Herbst). Proceedings of the 6th International Working Conference on Stored-product Protection – Volume 1: 221-225.

**Winks, R. G. 1984.** The toxicity of phosphine to adults of Tribolium castaneum (Herbst): Time as a dosage factor. J stored Prod Res. 18, 159-169.

**Winks, R. G. 1985.** The toxicity of phosphine to adults of Tribolium castaneum (Herbst): phosphine-induced narcosis. J Stored Prod Res. 21: 25-29.

**Table 4-1. The mean knockdown time (KT) in minutes for populations of adult *Rhyzopertha dominica* tested as single beetles or as groups of 10 for knockdown of 50% or 100% of the group.**

[1]Populations listed and their corresponding FAO % Resistance values are taken directly from table 2-1 in Chapter 2.

| Population[1] | FAO % Resistance[1] | Single KT (± SE)[2,3] | 50% KT (± SE)[2,3] | 100 % KT (± SE)[2,3] |
|---|---|---|---|---|
| USDA | 0 | 6.01 ± 0.13 O, b | 5.91 ± 0.32 F, b | 10.41 ± 0.52 F, a |
| Carnduff | 0 | 7.54 ± 0.14 N, b | 6.46 ± 0.14 EF, b | 14.35 ± 0.73 F, a |
| Coronach | 0 | 11.09 ± 0.38 L, b | 10.38 ± 0.80 CDEF, b | 17.37 ± 0.15 F, a |
| Lethbridge | 4 | 9.10 ± 0.11 M, b | 8.11 ± 0.80 DEF, b | 13.86 ± 0.35 F, a |
| Stirling | 14 | 11.00 ± 0.17 L, b | 12.39 ± 0.62 CDEF, b | 28.09 ± 4.13 EF, a |
| Williams | 53 | 14.35 ± 0.34 K, b | 13.03 ± 0.70 CDE, b | 60.26 ± 3.17 DE, a |
| Victoria | 67 | 17.56 ± 0.22 J, b | 15.70 ± 0.41 BC, b | 72.89 ± 4.32 D, a |
| Princeton | 71 | 19.34 ± 0.18 I, b | 11.47 ± 0.29 CDEF, c | 88.14 ± 0.58 CD, a |
| Parlier 2 | 72 | 19.66 ± 0.16 I, b | 12.53 ± 0.54 CDE, c | 77.19 ± 0.90 D, a |
| Garden City | 74 | 19.52 ± 0.33 I, b | 14.59 ± 0.32 CD, c | 97.07 ± 2.32 CD, a |
| Clifton | 80 | 21.68 ± 0.11 H, b | 14.97 ± 0.68 CD, b | 96.45 ± 16.31 CD, a |
| Burleson | 87 | 25.09 ± 0.23 G, b | 12.75 ± 0.60 CDE, c | 134.06 ± 7.30 C, a |
| Belle Glade | 87 | 59.52 ± 0.27 A, b | 41.31 ± 5.29 A, c | >300 A, a |
| Uniontown | 89 | 31.79 ± 0.39 D, b | 13.85 ± 0.48 CD, c | 104.50 ± 1.13 CD, a |
| AB1 | 93 | 30.24 ± 0.23 E, b | 14.27 ± 1.10 CD, b | 125.12 ± 20.77 C, a |
| AB2 | 90 | 26.44 ± 0.32 F, b | 12.96 ± 0.51 CDE, c | 101.19 ± 11.64 CD, a |
| Minneapolis | 97 | 57.01 ± 0.70 B, b | 21.62 ± 0.81 B, c | 211.43 ± 7.50 B, a |
| Parlier 1 | 97 | 55.65 ± 0.21 C, b | 20.98 ± 0.40 B, c | 175.78 ± 26.18 B, a |

[2]Means for KT results in a row followed by the same lower-case letter, and those in a column followed by the same upper-case letter, are not significantly different according to a REGWQ post hoc analysis.

[3]All differences determined by an ANOVA: Population – $F_{range}$= 25.11-14219.10, $p<<0.01$ and KT- $F_{range}$ =61.32-3300.98, $p<<0.01$

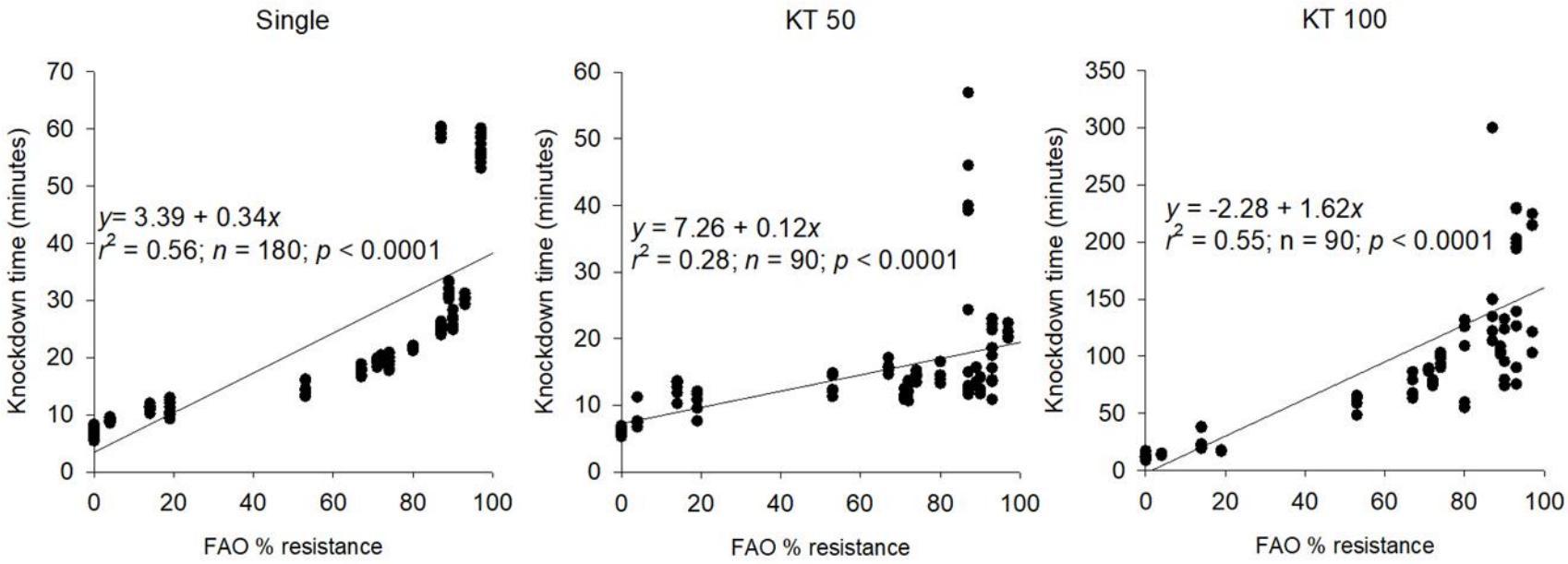


**Figure 4-1. Regression of knockdown times as a function of pre-determined FAO discriminating dose resistance frequencies for raw data on individual beetles or groups as reported in Table 2-1**

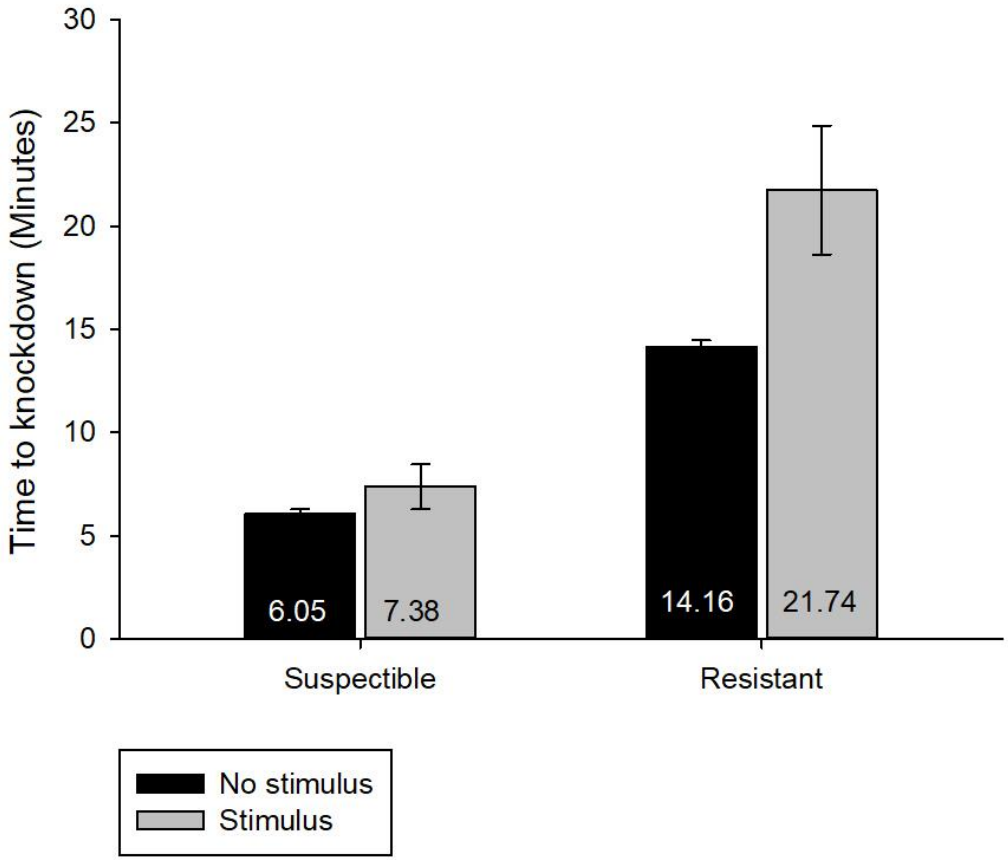


**Figure 4-2. Mean (SE) time to knockdown for individual *Rhyzopertha dominica* from susceptible and resistant populations that were either given a physical stimulus or not while being exposed to 3000 ppm phosphine concentration.**

p-value susceptible << 0.01, p-value resistant < 0.03

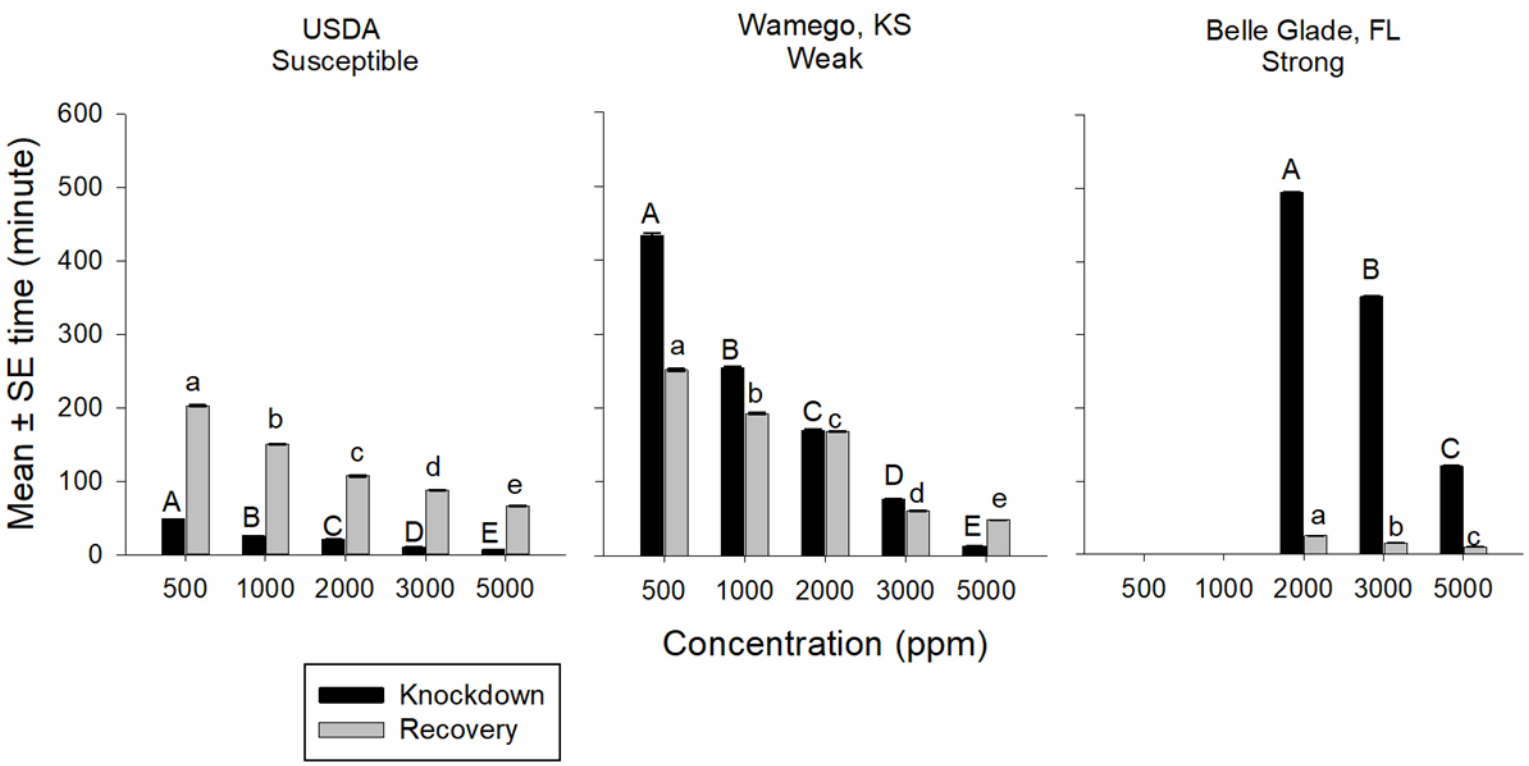


**Figure 4-3. Mean (SE) time to knockdown and recovery time for group of 10 adult *Rhyzopertha dominica* from susceptible, weak and strong resistant populations exposed to different phosphine concentrations**

KT – $F_{range}$= 3786.56-200026, p<<0.01 and RT- $F_{range}$ =144.33- 10709.5, p<<0.01