

PHOSPHINE RESISTANCE IN NORTH AMERICAN *TRIBOLIUM CASTANEUM* (HERBST)

(COLEOPTERA: TENEBRIONIDAE)

by

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B.S., Arkansas State University, 2013

A THESIS

submitted in partial fulfillment of the requirements for the degree

MASTER OF SCIENCE IN ENTOMOLOGY

Department of Entomology  
College of Agriculture

KANSAS STATE UNIVERSITY  
Manhattan, Kansas

2015

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## Abstract

Resistance of stored-grain insects to the fumigant gas phosphine is becoming common, due to genetic-based resistance. Using proper resistance management, phosphine can continue to be effective with efficient monitoring methods. This thesis focuses on assessing the levels of phosphine resistance across North America in a common stored-product pest, the red flour beetle, *Tribolium castaneum* (Herbst), and on refining a rapid bioassay method so that resistance can be easily and accurately detected.

Previous work found that phosphine resistance was present in two regions of the USA, the Southeast and Midwest. In this study a discriminating-dose bioassay was used with adult beetles to determine the number of resistant and susceptible individuals in a test population. Adult *T. castaneum* from 25 locations across the United States and Canada were collected, and 25-60 adults from each population were assessed for the percentage of resistant individuals. Thirteen populations were deemed susceptible with no resistant insects in the samples, while eight populations had 52% or fewer individuals scored as resistant, and four populations had between 88 and 100% resistant individuals. Dose-mortality experiments were conducted to characterize the “strength” of resistance. One population with 41% resistant beetles in the discriminating dose assay was 4.5-fold resistant relative to the susceptible laboratory strain, compared to 127-fold resistant for a population with all 100% scored as resistant. Adult beetles from twelve populations were used to determine if a “knockdown” test of insects exposed to a high concentration of phosphine (3000 ppm) could assess resistance as well as the discriminating-dose mortality assay. The time required for five out of ten beetles to be knocked down, called the  $KT_{50}$  for the time to knockdown 50% of the sample, was useful to characterize resistance. Phosphine susceptible beetles had  $KT_{50}$  values less than 15 minutes, while samples

from resistant populations had  $KT_{50}$ 's between 15 and 52 minutes. The refined quick test and knowledge of current levels of phosphine resistance in the United States and Canada reported in this thesis point to the importance of such information in developing phosphine resistance management programs for grain insects.

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## Acknowledgements

There are many people to thank for their help in my completion of this degree. First, I would like to thank my advisor Dr. Thomas Phillips for not only taking me on as a student, but also for spending countless hours of his time in helping me with this degree. Next, I would like to thank my committee members, Dr. Frank Arthur and Dr. Bhadriraju Subramanyam for their help and advice during my time at K-state. I would also like to thank Dr. John Ruberson for putting up with my countless inquiries for what must have seemed like the longest two years of his career. There are also many members of Dr. Phillip's laboratory that helped me with my thesis work on a daily basis: Jaime Aikins, Dr. Zhaorigetu Chen, Barbara Amoah, Salehe Abbar, Dr. Mukti Ghimire, and Dr. Ozgur Saglam. Special thanks is for Stephen Losey, Edwin Afful, and Erick Cordeiro who spent countless hours putting up with me and helping me with experimental design and data collection. I am very grateful for the samples of live beetles sent from many locations by helpful colleagues and industry contacts. I would also like to thank my sources of funding, CRC Plant Biosecurity and the Kansas State Department of Entomology. Included also are my thanks for a wonderful department full of some of the most helpful colleagues and staff that I have ever had the pleasure to meet.

Alongside the wondrous amount of thanks I have for wonderful colleagues, I must also thank my family. Thanks first and foremost to my best friend and wife Sarah Cato, who was here with me every step of the way. I would also like to thank my parents Ricky Cato and Melissa Kitchens. Without their wise knowledge and guidance I would not be here today. Finally, I would like to also thank my siblings, Chris, Daniel, and Megan, who were always there when I needed them to be.



# **Chapter 1 - Literature Review**

## **Introduction**

The red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is one of the most common pests found in stored-grain environments (Cuperus et al. 1986). This pest also infests the majority of stored-product materials (Hagstrum and Subramanyam 2009). Due to their presence in most stored-product environments, *T. castaneum* populations are controlled or managed in many different ways including: sanitation, residual insecticide applications, and fumigation. This pest is well known to adapt to poorly administered controls and develop resistance to many different types of insecticides with different modes of action (Zettler 1991).

One of the most widely used fumigants throughout much of the world is hydrogen phosphide, also known as phosphine (PH<sub>3</sub>). This fumigant is effective as it is poisonous to all stored-product insects and all of their life stages, very easy to apply in tablet formulations that release phosphine by reacting with moisture in the air, and it is a gas that can spread evenly throughout a stored-grain environment (Chaudhry 1997). However, because of misuse through poorly sealed grain environments along with key resistant mutations that can be selected by phosphine fumigation (Chaudhry 1997), phosphine resistance has started to cause failed fumigations which could lead to the loss of one of few effective tools for pest management in stored grain.

Knowledge of phosphine resistance has led to the development of discriminating dose bioassays to assess levels of resistance in many different countries (Food and Agriculture Organization 1975). However, these bioassays are technical to conduct and are not viable for use by grain managers or pesticide applicators due to the long time, training, and expensive equipment needed for evaluation. Grain managers with grain containing phosphine-resistant insects cannot tolerate the high costs of a failed fumigation. The majority of grain facilities are not aware that phosphine resistant pests may occur in storage.

The purpose of this study was to assess the variation in among populations of *T. castaneum*. A discriminating dose bioassay was used to assess whether resistance was present or absent in a population. A dose response bioassay was also performed on susceptible, weak, and strongly resistant populations to have a better understanding of what percent resistance means. Work was also done to assess the viability of a narcosis-based knockdown assay, and what methods would best lead to its use by grain managers and pest controllers.

### **The Red Flour Beetle, *Tribolium castaneum* (Herbst)**

*Tribolium castaneum* is a cosmopolitan pest that is known to infest 246 commodities worldwide (Hagstrum and Subramanyam 2009), although it prefers flour and other milled products (Good 1936). *T. castaneum* is a dark reddish brown beetle that averages 3.3 mm in length (Good 1936). Eggs are oblong and are of a whitish/colorless translucent complexion. These eggs are covered with a substance that allows them to adhere to the substrate they are laid in, which is typically a food substrate for both adults and larvae (Good 1936). The incubation period for the eggs was tested at different temperatures by Good (1936), and it was found that the optimum temperature was likely around 30°C with eggs hatching in 3-5 days. Good (1936) also showed that as temperature decreased to 25°C, the average time it took for an egg to hatch increased to six days.

Larvae are elongate and cylindrical with a mostly white complexion, and they take from 22-100 days to develop depending on the food source and other environmental conditions (Good 1936). Larvae typically have seven instars, but this can range anywhere from 5-11 instars depending on the environmental conditions and variation within individuals (Arbogast 1991). Once the larvae have matured and are ready to pupate they come to the surface of the grain and spend anywhere from six to twelve days pupating (Good 1936). Adult individuals are considered

long lived pests and some beetles have been observed living up to three years. Once they have eclosed from the pupal casing, they begin mating one to two days after emergence. Adults will oviposit for an average of 148 days at 27°C giving them an average fecundity of 327 eggs/female at this temperature (Good 1936).

*T. castaneum* is known to produce defensive compounds called quinones from its odoriferous glands (Alexander and Barton 1943). These compounds are not unique to this species, as they are common in much of the tenebrionid family. These secretions are potentially toxic to the beetle itself (Roth and Howland 1941) and lead to the contamination of any foodstuff they are associated with during their lifetime (Ogden 1969). Flour that has been heavily contaminated by these beetles gives off a pungent odor and has a pinkish tint, with lowered quality (Payne 1925). These beetles also produce an aggregation pheromone that is released by the males and attracts members of both sexes (Suzuki 1981). This aggregation pheromone has been used in conjunction with pitfall traps as a means to monitor this pest's movement within mills and other facilities (Barak and Burkholder 1985).

*T. castaneum* is a highly mobile species that feeds on stored-products during both the larval and the adult stage. Adults are considered long lived and will mate during the majority of their life as adults, leading to a long-lived and exponentially growing population (Good 1936). In specific environments such as stored grain, *T. castaneum* are one of the most commonly found pests (Cuperus et al. 1986). Because of these implications, control of this species is paramount to successful grain storage.

## **Phosphine**

Hydrogen phosphide gas (PH<sub>3</sub> or Phosphine) is one of the most commonly used fumigants and it has virtually replaced a wide variety of previously used fumigants (Halliday et

al. 1983). This fumigant was popularized quickly due to its ability to be rapidly diffused in air (Weast 1987), and after the development of metal phosphide formulations, gas was able to be released within the bins (*in situ*) or other areas of use on exposure to moist air (Heseltine and Thompson 1957; Bond 1984). Also, unlike other pesticides on grain, phosphine will not negatively affect seed viability (Strong and Lindgren 1960; Zutshi 1966; Ahmad 1976; Sittisuang and Nakakita 1985; Krishnasamy and Seshu 1990) or leave toxic residues on stored-products if properly used (Bruce et al. 1962; Scudamore and Goodship 1986; Lee et al. 1991). Although the ease of handling and effectiveness of phosphine has led to its widespread use, phosphine gas is corrosive to many metals including silver, gold, and most importantly copper, especially at high humidity (Bond et al. 1984). Because of its corrosiveness to copper, phosphine is not normally used in mills and other facilities containing electrical equipment.

Phosphine is generally used in the form of pellets, tablets, and sachets of metallic formulations, aluminum or magnesium phosphide, which allow the gas to be released when contacting moist air or moisture from grain and other commodities at proper temperatures (Bond 1984). These pellets, tablets, and sachets also contain ingredients that keep phosphine from combusting, as it is capable of violently exploding if the concentration reaches 18,000 ppm. Phosphine pellets are typically spread evenly throughout a grain mass or other types of commodity. The pellets, tablets, or sachets then react with water vapor in the air of a humid environment, or from the moisture from the grain itself. Before phosphine pellets are added, sealing of the space that is to be fumigated is key for effective use (Bond 1984). Phosphine is a very toxic fumigant to stored-product insects, but it 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). This fumigant is considered to be

highly toxic to stored-product insects, but the exact mode of action of phosphine is still much of a mystery. It is proposed by Nath et al. (2011) that phosphine likely has three modes of action: disruption of the sympathetic nervous system, suppressed energy metabolism, and toxic changes to the redox state of the cell. Although with other fumigants it is possible to decrease the exposure time of a fumigation by raising the concentration of the fumigant added, many stored-product insects become more tolerant to phosphine as the concentration is raised due to a narcotic effect, making the relationship of concentration and time nonlinear (Winks 1974).

Phosphine has always required a long exposure time in very tightly sealed spaces, but these standards are a rare occurrence in its actual use (Chaudhry 1997). If during the fumigation the concentration falls quickly due to leaks in the structure, insect populations will generally not be fully controlled and pest populations can recover quickly from any incomplete control and actions with phosphine (Chaudhry 1997). If these sub lethal exposures are repeated frequently, which is common in many areas of the world such as Oklahoma where it is used in every wheat storage facility at least once a year (Flinn et al. 2003), selection of phosphine resistant individuals is possible (Chaudhry 1997).

Three levels of phosphine susceptibility or resistance can be characterized: susceptible, weak, and strong resistance (Collins and Emery 2002), and these classifications can be considered phenotypes resulting from key resistance genes (Schlipalius et al. 2012). If bins are sealed properly and the label rate of phosphine is used, weakly resistant and susceptible *T. castaneum* can likely be controlled. Once beetles reach up to 80% resistance, it is possible for beetles to gain strong resistance and no longer be controlled despite proper sealing of bins (Collins and Emery 2002). The phenomenon of strong resistance was confirmed to exist in *T. castaneum* (Emery et al. 2011). The mechanism of resistance is likely due to 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). Because the conditions are many times favorable for phosphine resistance, a survey of resistance in every area that has high phosphine use or grain movement should be conducted so that proper resistance management techniques can be followed.

The first survey on the possible resistance of phosphine and four other commonly used pesticides was performed during 1972-1973 (Champ and Dyte 1976). This was a worldwide survey that found that at least 10% of the sampled populations were not 100% susceptible to phosphine when compared to a susceptible population at the same dosage. Included in these populations were many from the United States that showed some resistance, although at most only a few individuals were capable of surviving the discriminating dose bioassay (Champ and Dyte 1976). Although not much research was done in the United States in the immediate aftermath of these findings, research continued in many other parts of the world.

A few years after the first world-wide survey by Champ and Dyte (1976), failed fumigations were increasing in prevalence in Bangladesh and resistance was found in areas of Australia. Attia (1983) found that around 10% of the *Tribolium castaneum* strains collected from areas of Australia were resistant to phosphine, with many other species of beetles showing some prevalence of resistance. Using dose responses compared to susceptible populations, Mills (1983) found that *Tribolium castaneum* adults from Bangladesh were sixteen times more tolerant to phosphine than that of a susceptible population, denoting significant resistance. These results were corroborated by another group that also found resistance in Bangladesh and published the same year (Tyler et al. 1983). These locations that harbored resistance were found to have non-airtight areas of fumigation, likely leading to the resistance (Mills 1983). An earlier study

dealing with *Trogoderma granarium*, the khapra beetle Everts, found a resistant strain in Punjab in 1979, but this study did not feature *Tribolium castaneum* (Borah and Chahal 1979).

Further international surveys on phosphine continued, especially when insect problems became noticeable after areas reported failed fumigations. From 1986-1988 a survey in the state of Sao Paulo, Brazil found that of the ten *T. castaneum* strains that were tested for phosphine resistance, nine were found to be resistant (Pacheco et al. 1990). Along with the resistance that was observed in *T. castaneum*, resistance was also found in three of four other species at around the same rate (Pacheco et al. 1990). Due to reports of failed fumigations in Morocco, from June-July of 1999, insects from many wheat storage facilities were tested for phosphine resistance (Benhalima et al. 2004). Of the 51 populations of three different insect species, 50 of them were found to be resistant to phosphine. The majority of these populations, especially the populations of *R. dominica* and *T. castaneum*, showed survival from 50-80%. This indicated possible high levels of resistance and poor fumigation techniques in a country that almost solely relies on phosphine (Benhalima et al. 2004). Of the ten strains of *T. castaneum*, all of them were resistant, with the highest percent mortality being between 50-60%, and the lowest at 10% mortality. This is likely an indication of populations that will not be able to be controlled with phosphine without perfect fumigations every time (Benhalima et al. 2004). Many other surveys were performed throughout much of the world where phosphine is heavily relied upon (Attia and Greening 1981; Champ 1985; Udeaan 1990; Chaudhry 1991; Taylor 1991; Irshad et al. 1992; Udeaan 1992; Rajendran and Narasimhan 1994; Bell and Wilson 1995; Sayaboc et al. 1998; Emery et al. 2011; Lorini *et al.* 2011).

Although phosphine monitoring has been prevalent in many parts of the world since the Champ and Dyte (1976) survey, the United States has seen very little research. The first survey



of phosphine resistance in the United States was done by Zettler et al. (1989) with strains collected in 1986. In this study three pest species were the main focus in peanut storage facilities in Georgia, Florida, and Alabama. Of the eighteen strains of *T. castaneum*, eight of these were found to be resistant using a discriminating dose bioassay similar to the FAO bioassay (Food and Agriculture Organization 1975). Only one of these strains exhibited resistance with a medium amount of survival, at 36%. Most of the populations were significantly different than the known susceptible population, but survival was around 5% (Zettler et al 1989). Although this was the first survey of phosphine resistance in the United States, however the focus of this study was only on one commodity and in three states with the sites fairly close together.

The second survey of phosphine resistance was published one year later and was performed in ten counties in northwestern & central Oklahoma (Zettler and Cuperus 1990). For this survey only two species were assayed, *T. castaneum* and *R. dominica*. Of the nine populations of *T. castaneum*, only one was considered resistant, with 94% mortality. The *R. dominica* populations showed a higher prevalence of resistance with 8 of 21 populations showing resistance, but in their levels of resistance they far out stretched *Tribolium* with mortality as low as 8% in one strain (Zettler and Cuperus 1990). From this survey it is clear that some species have garnered resistance in the central United States, but this study is still limited in the geographic area that it covers. From 1973-1990 only two small areas of the United states have been surveyed for resistance, with data on much of the rest of the country being absent.

The most recent survey in the United States on *Tribolium castaneum* was conducted from 2010-2011 using insects collected from grain storage locations in Oklahoma (Opit et al. 2012). In this survey nine different populations of *T. castaneum* and five different populations of *R. dominica* were assayed for phosphine resistance. This survey was meant to revisit areas near

where Zettler and Cuperus surveyed insects in 1990 to better show the progression of resistance in the areas. For *T. castaneum*, eight of the nine populations were found to be resistant to phosphine, with survival being as high as 94% in one population. All of the five populations of *R. dominica* were found to be resistant, with survival being as high as 97% percent, and three of the five populations had survival over 90% (Opit et al. 2012). Once the levels of resistance were determined using the discriminating dose bioassays, populations with high survival were then assayed using a dose response to determine how resistant the individuals were. One population of *T. castaneum* was determined to be around 21 times resistant than the susceptible strain according to the  $LC_{50}$ , and 3 strains of lesser grain borer were found to be around 95, 161, and 443 times more resistant than the susceptible strain according to the  $LC_{50}$  (Opit et al. 2012). These results indicate that both species of these stored product pests would be very hard to control using phosphine without using resistant management techniques, especially if bins are imperfect and allow leaks.

Although another phosphine resistance survey has been done within the United States, it was only on one other species of stored product pest, *Lasioderma serricorne*, the cigarette beetle, in the southeastern united states, the same area where Zettler et al. assayed three species in 1989 (Zettler and Cuperus 1990; Zettler and Keever 1994). Much of the United States grain industry is likely using phosphine in the same frequency as in Oklahoma where surveying has been completed, so phosphine resistance could be hypothesized to be present (Flinn et al. 2003). Because of the possible presence of phosphine resistance in many of these areas it is imperative that surveys be completed so that proper resistance management techniques can be employed to save the usage of one of the easiest and most efficient fumigants available for use right now. Not only should resistance surveys be completed on populations throughout the United States,

Canada has no data published on phosphine resistance since Champ and Dyte (1976) even though it is likely used for control in these storage areas. Along with resistance surveys, dose response assays are needed to better understand percent resistance according to the discriminating dose assays, to help gauge the usefulness of a good fumigation of phosphine for specific populations.

In Australia, it has been determined that these surveys of phosphine resistance are important for proper resistance management, and have become a mainstay (Emery et al. 2011). Included in Australia's management of high levels of phosphine, increased exposure periods are being used in tandem with properly sealed bins based on the knowledge of the resistance in these particular areas (Collins et al. 2001). To better understand the situation in each area where resistance could be located, more rapid measures to survey have been deemed necessary for proper management (Nayak 2012). With this knowledge a proper plan is created and implemented, with alternatives such as sulfuryl fluoride being used when strong resistance that likely cannot be controlled by phosphine is discovered (Nayak et al. 2010).

### **Methods of Monitoring Phosphine Resistance**

Currently the most widely used and accepted test for phosphine resistance in many stored-product pests is FAO method number 16 (Food and Agriculture Organization 1975). This method uses a discriminating dose bioassay with a concentration based on the LD<sub>99.9</sub> for seven different pests of stored grain, including *T. castaneum*. Insects are exposed to phosphine for twenty hours at 25°C and then maintained in air for fourteen days at 25°C to allow for delayed mortality or recovery. Fourteen days after the end of the exposure, survival of the insects is assessed, with 100% mortality as an indication of a fully phosphine susceptible population (Food and Agriculture Organization 1975). The inherent issue with using this method, and a mortality

based method, is the fifteen days that it takes to determine resistance. Because of this, more rapid test avenues have been explored.

Using newer genetic methods, research has found that it may be possible for certain levels of phosphine resistance to be identified using PCR and molecular markers (Chen et al. 2015). In 2012, one gene mutation that led to strong resistance in both *Tribolium castaneum* and *Rhyzopertha dominica* was identified by Schlipalius et al. (2012). A point mutation in a core metabolic enzyme, dihydrolipoamide dehydrogenase (DLD), was identified to lead to strong resistance in these species, but mutations at other genes are likely to code for resistance as well, particularly the weak phenotype. (Schlipalius et al. 2012). Using this point mutation, a test that works much more quickly than the fifteen day FAO standard test was created to rapidly identify populations that likely cannot be controlled by normal fumigations (Chen et al. 2015). This is an interesting avenue and a great improvement in the ability to identify resistance, but this method still requires experienced laboratory technicians and expensive equipment to yield results. This coupled with the fact that it can't identify incipient resistance problems, leaves us in a situation that is similar to what the FAO provides versus what is desperately needed.

One approach that has been explored for possible “quick tests” is the use of a narcotization (knockdown) behavior found in many pests when exposed to phosphine (Bang and Telford 1966). When insects are exposed to phosphine at a high concentration (around 220-360 ppm or higher) they will enter a narcotized state where the majority of the insects will be able to recover if exposure times are not long. Bang and Telford (1966) also found that as the concentration of phosphine was increased, the time to narcotization decreased. Because of this correlation, researches assessed whether insects that were resistant to phosphine according to the FAO method also showed an increased time to knockdown. Reichmuth (1991; 1994) found that

resistant *R. dominica* took longer to be narcotized than susceptible beetles of the same species, which he later attributed to a lowered uptake of phosphine in resistant beetles. Waterford and Winks (1994), using a phosphine susceptible population, compared the knockdown time of individuals before doing specific crossing with individuals based on their varying time-to-knockdown. They found that the more knockdown resistant cross had a higher knockdown time when compared to crosses of the most susceptible individuals after twenty generations. The same correlation was found with the knockdown of the population vs. crosses of the most knockdown resistant populations, as knockdown times were greatly increased. This study, along with the previous, showed that a phosphine resistance test using time-to-knockdown was viable because resistant populations took longer to knockdown than susceptible populations (Waterford and Winks 1994).

The first such test that was created to replace the lengthy FAO test was that of Reichmuth's (1991). In this test *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 period of time the population was deemed resistant (Reichmuth 1991). This quick test only contained two susceptible strains and one resistant strain for comparison, and used a wide range for dosage. This range of dosage was used because Reichmuth (1991) determined that above 700 ppm, concentration was not a factor in knockdown time of the two susceptible strains. This is contrary to what Waterford and Winks (1994) found, as they found 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. For the concentration that Reichmuth (1991) suggests no difference in knockdown time (above 700ppm), Winks (1985) found knockdown times from

26.3-8.9 minutes (714-3427ppm respectively) in a susceptible strain. Although Winks (1985) only looked at 3 concentrations in the range of around 1000-3000, a difference looks evident in 1000ppm and 3000ppm. From this it could be assessed that a stable, quantifiable concentration may be necessary when looking for presence of resistance or comparing strains using the knockdown method.

Beyond the first true quick test created in 1991 by Reichmuth, many other researchers have attempted to create a shortened identification of phosphine resistance. Bell et al. (1994) created a quick test method to determine a discriminating time between resistant and susceptible strains as indicated by the FAO method. This study used similar methods as Reichmuth, but a range of 221-299 ppm (Bell et al. 1994) was used as opposed to much higher concentrations favored by Reichmuth (1991). These concentrations are on the lower end those that initiate narcosis, but Bell et al. (1994) showed an ability to discriminate between resistant and susceptible species using their methods.

Another quick test was assessed using very similar methods at a concentration of 1428 ppm (Cao and Wang 2000). This quick test was created by looking at the correlation of the LC<sub>50</sub> and KT<sub>50</sub> between both resistant and susceptible strains of three different species including *Tribolium castaneum* (Cao and Wang 2000). They then created a variation of Reichmuth's 30 minute knockdown test using KT<sub>50</sub> instead of KT<sub>100</sub>, and a set concentration of phosphine (Cao and Wang 2000; Reichmuth 1991). With this test they were confident that they could predict susceptibility within 30 minutes of fumigation. It is of note that both this quick test and the test created by Bell et al. defined knockdown as the insects being hampered from walking in a normal fashion due to exposure to phosphine gas. Cao and Wang (2000) mentioned in their publication that Reichmuth used the parameters of "walking normally" to define a resistant

individual and then used those parameters for their own test. Reichmuth (1991) actually defined knockdown as the insects remaining motionless and then later stated that insects that were active and not knocked down were deemed resistant.

The only quick test to actually be made into a kit for commercial use was created by Steuerwald et al. (2006), which was later derived into a prototype test kit (Degesch Inc. <http://www.degeschamerica.com>). This quick test is similar to the previously developed tests and uses 3000 ppm and a fixed amount of exposure time to judge the susceptibility of populations (Steuerwald et al. 2006). Populations are scored after a fixed amount of time based on whether or not beetles are “active” within the syringe containing phosphine (Steuerwald et al. 2006). Although this was presented at the 9<sup>th</sup> International Working Conference for Stored-products, nine years later it still is not being used commercially to identify phosphine resistance.

Although many of these quick tests have aspects that lead to a commercially-viable test, they are also lacking in many forms. First, all of these methods looked at hampered movement with little explanation of what hampered movement actually means. Steuerwald et al.’s (2006) test explains knockdown as inactivity, Reichmuth (1991) defines knockdown as the insects remaining motionless but gives no parameters, and Bell et al. implies knockdown as the insect’s inability to walk up a cone of paper (1994). Commercial quick tests will need methods that are extremely easy to follow and not left up to the interpretation of each user. Second, these proposed tests either use  $KT_{100}$  or  $KT_{50}$  as the parameter to denote presence or absence of resistance.  $KT_{100}$  is likely used in many of these tests because of the comparison to the discriminating dose bioassay (FAO) that is the mainstay in phosphine resistance monitoring. In personal observations it became increasingly obvious that end point knockdown is widely variable for some strains, whereas the first 50% of a sample become knocked down much more

uniformly and are unlikely to recover during the fumigation (which was observed countless times in  $KT_{100}$  tests). Last, all of these tests except for the commercialized version use sophisticated methods to generate phosphine gas for fumigations. Although these are easy to do in a laboratory setting, grain managers will actually need to use something similar to what Steuerwald et al. (2006) have created in a kit form, which is why further research on quick tests should keep this potential commercially-viable test kit in mind.



## Objectives:

Chapter 2 compares the variation of phosphine resistance among *T. castaneum* populations on a geographic scale. The first objective of that study was to assess the levels of resistance found from samples throughout the United States and parts of Canada. This study was done on two scales: populations found throughout the north-central region of Kansas, and all strains collected from other parts of North America. The second objective of this study was to perform dose response assays on populations that fit into three possible groups of phosphine resistance: populations susceptible to phosphine, populations with a low frequency of resistance to phosphine, and populations with a high frequency of resistance to phosphine. This objective should help better explain what exactly the percent resistance determined by the FAO discriminating dose bioassay actually means to the management of a population. The third objective was to test populations of *T. castaneum* currently held in culture since 2011 for resistance and compare that to insects collected from those same sites in 2014. This comparison should show whether or not phosphine resistance is stable in populations with no pressure for extended periods of time, and if resistance in a newly collected sample from the same geographic location is increasing, decreasing, or staying the same.

The overall goal of Chapter 3 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. The first objective of this study was to assess three different quick test bioassays for knockdown time (KT):  $KT_{100}$ ,  $KT_{50}$ , and knockdown of single individuals, to predict the FAO frequency of resistance in a given population. This was done by comparing the time to knockdown of many strains with the percent resistance assessed by the FAO standard test using an exact definition of knockdown. Also in this study, the disturbance of quick test environments (and the beetles inside them) was assessed to see if it affected knockdown time

during single insect trials, to possibly explain why single insects never stay active as long as insects within a group.

## References

- Ahmad, M. 1976.** Effect of phosphine on the germination of edible legume seeds. *Journal. Stored Prod. Res.* 12: 211-212.
- Alexander, P. and D. H. R. Barton. 1943.** The excretion of ethyl quinone by the flour beetle. *Biochem. F.* 37: 453-465.
- Arbogast, R. T. 1991.** Beetles: Coleoptera, pp.131-176. *In* J. R. Gorham, [ed.], *Ecology and management of food-industry pests.* Assoc. Offic. Anal. Chem., Arlington.
- 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.
- 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.
- 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 W. E. Burkholder. 1985.** A versatile and effective trap for detecting stored-product Coleoptera, pp, 207-218. *In* A.V. Barak and W. E. Burkholder [eds.], *Agriculture, Ecosystems and Environment.* Elsevier Science Publishers B.V., Amsterdam.
- Bell, C.H., N. Savvidou, K. A. Mills, S. Bradberry, and M. L. Barlow. 1994.** A same-day test for detecting resistance to phosphine, pp 41-44. *In* Proceedings of the 6<sup>th</sup> International Working Conference on Stored-product Protection. 17-23 April, Canberra, Australia. CAB International, Wallingford, United Kingdom.

- Bell, C. H., & S. M. Wilson. 1995.** Phosphine tolerance and resistance in *Trogoderma granarium* Everts (Coleoptera: Dermestidae). *Journal. Stored Prod. Res.* 31: 199-205.
- 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. *Journal. Stored Prod. Res.* 40: 241–249.
- 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. *Journal of Stored Prod. Res.* 20: 57-63.
- Borah, B. and B. S. Chalal. 1979.** Development of resistance in *Trogoderma granarium* Everts to phosphine in the Punjab. *FAO Plant Prot. Bull.* 27: 77- 80.
- Bruce, R. B., A. J. Robbins, and T. O. Tuft. 1962.** Fumigant Residues, Phosphine residues from Phostoxin-treated grain. *J. Ag. Fd. Chem.* 10: 18-21.
- 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.
- Champ, B. R. 1985.** Occurrence of resistance to pesticides in grain storage pests. Pesticides and humid tropical grain storage systems (Ed. BR Champ, E Highly), pp. 229-255. *In* Australian Centre for International Agricultural Research Proceedings 14.

- Chaudhry, M. Q. 1991.** Biochemical mode of action of phosphine and mechanisms of resistance in two species of stored-product beetles. Doctoral dissertation, University of Reading. 180 pp.
- 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.
- 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.
- Collins, P. J., G. J. Darglish, M. K. Nayak, P. R. Ebert, D. Schlipalius, W. Chen, H. Pavic, T. M. Lambkin, R. Kopittke, and B. W. Bridgeman. 2001.** Combating resistance to phosphine in Australia, pp. 593-607. *In* Int. Conf. on Controlled Atmosphere and Fumigation in Stored Products, 29 October – 3 November, Fresno, CA. Printco Ltd., Nicosia, Cyprus.
- Collins, P. J., G. J. Darglish, 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.
- 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. Kansas Entomol. Soc.* 59: 620-627.
- Emery, R. N., M. K. Nayak, and J. C. Holloway. 2011.** Lessons learned from phosphine resistance monitoring in Australia. *Stewart Posthar. Rev.* 7: 1-8.  
(doi:10.2212/spr.2011.3.8).

- Flinn, P. W., D. W. Hagstrum, C. Reed, and T. W. Phillips. 2003.** United States Department of Agriculture-Agricultural Research Service stored-grain area wide integrated pest management program. *Pest Man. Sci.* 59: 614-618.
- 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.
- Good, N. E. 1936.** The flour beetles of the genus *Tribolium*. *USDA Tech. Bull.* 5: 27-28.
- Halliday, D., A. H. Harris, and R. W. D. Taylor. 1983.** Recent developments in the use of phosphine as a fumigant for grain and other durable agricultural produce. *Chem. Ind.* 12: 468-471.
- Hagstrum, D. W. and B. Subramanyam. 2009.** Table 1.1 Insects and their natural enemies associated with stored products, pp 183. *In* Hagstrum, D. W. and B. Subramanyam (eds.), *Stored-product insect resource*. AACC International Inc. St. Paul, MN.
- Heseltine, H. K. and R. H. Thompson. 1957.** The use of aluminum phosphide tablets for the fumigation of grain. *Milling* 129: 676-783.
- Irshad, M., W. A. Gillani, and J. Iqbal. 1992.** Occurrence of resistance in stored grain pests to pesticides in Pakistan. *Pakistan J. Zool* 24: 79-82.
- Krishnasamy, V. and D. V. Seshu. 1990.** Phosphine fumigation influence on rice seed germination and vigour. *Crop Sci.* 30: 82-85.
- Lee, M. L., M. Y. Choi, and K. S. Choi. 1991.** Effects of controlling *Galleria mellonell* in honey bee combs with aluminum phosphide, its residues and safety to honey bees. *Korean J. Apic.* 6: 12-15.

- Lorini, I., P. J. Collins, G. J. Darglish, M. K. Nayak, 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.
- Mills, K. A. 1983.** Resistance to the fumigant hydrogen phosphide in some stored-product species associated with repeated inadequate treatments. *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 4: 98-101.
- 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>.
- Nayak, M., J. Holloway, H. Pavic, M. Head, R. Reid, and C. Patrick. 2010.** Developing strategies to manage highly phosphine resistant populations of flat grain beetles in large bulk storages in Australia, pp. 396-401. *In Proceedings of the 10<sup>th</sup> International Working Conference on Stored Product Protection, 27 June – 2 July, Estoril, Portugal.* Julius Kühn-Institut, Berlin, Germany.
- 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, Antalya, Turkey.* ARBER Professional Congress Services, Turkey.
- Ogden, J.C. 1969.** Effect of components of conditioned medium on behavior in *Tribolium confusum*. *Physiol. Zool.* 42: 266-274.
- 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.

- 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.
- Payne, N. 1925.** Some effects of *Tribolium* on flour. *J. Econ. Entomol.* 18: 737-744.
- Rajendran, S. and K. S. Narasimhan. 1994.** Phosphine resistance in the cigarette beetle *Lasioderma serricornis* (Coleoptera: Anobiidae) and overcoming control failures during fumigation of stored tobacco. *Int. J. Pest Manag.* 40: 207-210.
- 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 6<sup>th</sup> International Working Conference on Stored-product Protection*, 17-23 April 1994, Canberra, Australia. CAB International, Wallingford, United Kingdom.
- Roth, L. M., and R. B. Howland. 1941.** Studies on the gaseous secretion of *T. confusum* Duval. I. Abnormalities produced in *T. confusum* Duval by exposure to a secretion given off by adults. *Ann. Entomol. Soc. Am.* 34: 151-171.
- 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.
- Schlupalius, 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.



- Scudamore, K. A. and G. Goodship. 1986.** Determination of phosphine residues in fumigated cereals and other food-stuffs. *Pestic. Sci.* 17: 385-395.
- Sittisuang, P. and H. Nakakita. 1985.** The effect of phosphine and methyl bromide on germination of rice and corn seeds. *J. Pestic. Sci.* 10: 461-468.
- 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.*
- Strong, R. G. and D. L. Lindgren. 1960.** Germination of cereal, sorghum and small legume seeds after fumigation with hydrogen phosphide. *J. Econ. Entomol.* 53: 12-15.
- Suzuki, T. 1981.** Identification of the aggregation pheromone of flour beetles *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae). *Agric. Biol. Chem.* 45: 1357-1363.
- Taylor, R. W. D. 1991.** Resistance to grain fumigants and future prospects for their use. *Pestic. Outlook* 2: 22-24.
- Tyler, P. S., R. W. D. Taylor, and D. P. Rees. 1983.** Insect resistance to phosphine fumigation in food warehouses in Bangladesh. *Int. Pest Control* 25: 10-13.
- Udeaan, A. S. 1990.** Susceptibility status of *Trogoderma granarium* Everts populations to phosphine in Punjab. *Indian J. Ecol.* 17(2): 195-196.
- Udeaan, A. S. 1992.** Susceptibility status of *Trogoderma granarium* Everts populations to phosphine in north India. *J. Insect Sci.* 5: 217-218.
- 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 6<sup>th</sup>*

- 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, 67<sup>th</sup> edn. CRC Press Inc., Boca Raton FL.
- Winks, R.G. 1974.** Characteristics of response of grain pests to phosphine. Canberra, Commonwealth Scientific and Industrial Research Organization, Division of Entomology, Annual Report 1973-74.
- Winks, R. G. 1985.** The toxicity of phosphine to adults of *Tribolium castaneum* (Herbst): phosphine-induced narcosis. J. Stored Prod. Res. 21: 25-29.
- Zettler, J. L. 1991.** Pesticide resistance in *Tribolium castaneum* and *T. confusum* (Coleoptera: Tenebrionidae) from flour mills in the United States. J. Econ. Entomol. 84: 763-767.
- 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 D.W. Keever. 1994.** Phosphine resistance in cigarette beetle (Coleoptera: Anobiidae) associated with tobacco storage in the southeastern United States. J. Econ. Entomol. 87: 546-550.
- Zutshi, M. K. 1966.** Effect of aluminum phosphide (Phostoxin) on the germination of certain cereal and vegetable seeds. Bull. Grain Technol. 4: 197-199.

**Chapter 2 - Geographic Variation of Phosphine Resistance in the  
Red Flour Beetle, *Tribolium castaneum***

## Abstract

The red flour beetle, *Tribolium castaneum* (Herbst), is one of the most prolific stored product pests found throughout much of the world. This study focused on assessing the geographic variation of phosphine resistance in *T. castaneum* populations found across the United States and Canada using a discriminating dose bioassay. Along with these data, this study sought to perform dose-response assays on populations that have different frequencies of resistant insects that fit into groups of phosphine resistance. It was shown that resistance was present in previously surveyed areas, although with frequencies of resistance over 85% in many populations and even up to 100% in Red Level, AL. In the other two main areas assayed, four populations from Canada showed no resistance, and California contained mostly susceptible populations. However, resistance has been observed in California at 2 and 52%, meaning the genes coding for resistance are present and that intense surveying is necessary to fully understand resistance levels of the area. For the strong resistance strain used in the dose response bioassay, the resistance factor was over 100, showing a likely strongly resistant population. However, weakly resistant strains are likely to be controlled with proper fumigation techniques, at a resistance factor of only four. The results of this study suggest that proper resistance assessment techniques can help to identify phosphine resistance. Through surveying and the use of alternative fumigants on strongly resistant populations, phosphine can remain a mainstay in the stored grain environment.

## Introduction

Cereal grains can be stored for up to one year or longer after harvest (Hagstrum et al. 2012). During this time they can be under attack from many stored grain pests, including the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae), a common insect pest found in grain environments (Cuperus et al. 1986). This common pest is known to be resistant to many different pesticides including malathion, chlorpyrifos-methyl, and dichlorvos (Zettler and Cuperus 1990), and possibly because of this, grain storage control has moved mainly to the fumigant hydrogen phosphide (PH<sub>3</sub>), more commonly referred to as phosphine. Use of this fumigant has become widespread within its half-century of use, with states such as Oklahoma using phosphine at least once per year on all of its stored grain (Flinn et al. 2003). In environments such as wheat storage, phosphine is the only economically viable fumigant for removal of stored grain pests (Hagstrum et al. 1999). Phosphine is very important in the economics of stored grain because live insects can feed on the grain and cause extensive damage, and thus lower the quality and value at the point of sell.

Resistance to phosphine was first reported by Champ and Dyte (1976). After this report of increased tolerance to phosphine, resistance has been reported in many regions of the world (Borah and Chahal 1979; Tyler et al. 1983; Sayaboc et al. 1998; Benhalima et al. 2004; Lorini et al. 2007; Emery et al. 2011). Although there has been some work to look at phosphine resistance and its prevalence in the United States, there is still a lack of continuity in the understanding of the extent of resistance. Zettler and Cuperus (1990) found phosphine resistance in *T. castaneum* and *R. dominica* in wheat stored in Oklahoma in the late 1980s. Before that study was published, Zettler et al. (1989) had also showed some resistance levels in three different species of pests, including *T. castaneum*, attacking peanut storage facilities in Alabama, Georgia, and Florida. However, the only study after 1990 in the United States to attempt to characterize resistance was

that of Opit et al. (2012), who re-visited and found that resistance frequencies at some Oklahoma locations studied in the past were higher than those measured over twenty years earlier. Opit et al. (2012) used detailed dose-response assays on several populations and found levels of resistance of several hundred fold compared to a susceptible strain that would be difficult to control with current phosphine application rates.

The current status of phosphine resistance in the United States is currently only known in Oklahoma (Opit et al. 2012) and in Kansas (Chen et al. 2015). This fumigant is widely and almost exclusively used across the United States, thus knowledge of the variation on a geographic scale is important for continued use of phosphine for effective IPM programs. The first objective of this study was to survey sites from the US and Canada using a discriminating dose assay to determine presence or absence of phosphine resistance in *T. castaneum*. The second objective of this study was to perform dose-response assays fit into groups of phosphine resistance: populations susceptible to phosphine, populations with so-called weak resistance to phosphine, and populations with the reported strong resistance to phosphine. The third objective was to evaluate resistance frequencies in populations of *T. castaneum* held in laboratory culture since 2011 and compare these to insects collected from those same sites in 2014. This comparison should show whether or not phosphine resistance persists in populations with no selection pressure over years of laboratory rearing. It is hypothesized that there will be widespread resistance across much of the United States, especially in areas where commodity storage is prolonged. It is also hypothesized that populations that have been in a laboratory culture for three years will have similar levels of resistance to newly collected samples. With the geographic data that is reported from this survey, variation in resistance can be assessed, and this information can lead to improved IPM programs in regions containing resistance.

## Materials and Methods

### *T. castaneum* Acquisition and Rearing

Field-collected insects for this study were either brought into the laboratory directly, or if samples were very small (20 or fewer adults) they were allowed to propagate for adequate numbers for testing, or were historically sampled and reared within the laboratory for more than three generations. For all populations that were possible, laboratory colonies were maintained following initial evaluation with a standard protocol. Beetles were reared in Ball® 473 ml Wide Mouth Mason Jars, and a metal screen combined with filter paper was used instead of the supplied metal lid insert. These beetles were reared on organic golden buffalo flour supplemented with 5% brewer's yeast (95:5). Colonies were kept within growth chambers set at 28°C and 65% relative humidity with a photoperiod of 16hrs light and 8hrs dark. For most of the colonies used, many insects were available for use in both discriminating dose and dose response bioassays, but for a few samples received, colonies were started from only 20-30 insects (Goshen, CA, Walnut Hill, FL, and Tifton, GA).

Newly sampled populations were generally received in the mail following correspondence with many professionals associated with stored grain across North America including grain managers, commercial pest control operators, and academic colleagues. These samples were sometimes shipped as bulk grain samples from which beetles were removed in the lab, or samples of *Tribolium* adults with a small amount of grain were sent alone. Once samples were received the beetles were confirmed to species and used for discriminating dose bioassays. For other newly sampled populations, sites either nearby, or sites that had been historically assessed, were sampled for the possibility of *T. castaneum* presence. These sites were sampled weekly, bi-weekly, or monthly depending on the time of year and particular accommodation with

the grain storage owners. Typical on-farm grain bins were sampled using STORGARD® WB probe II® traps (TRÉCÉ, Adair, OK) with as many as eight traps being placed in a single grain bin. Commodities that these traps were used in were generally based on what the owner had available, and included wheat (*Triticum aestivum*), corn (*Zea mays*), and sorghum (*Sorghum bicolor*). For *T. castaneum* that were caught using these trapping procedures, beetles were placed in jars for rearing each week they were caught, with all *T. castaneum* from a specific population being placed in the same jar.

Table 1 reports the 25 populations studied here according to location information of province or state, city, and approximate GPS coordinates. Although many new populations were collected from across the North America, some populations of known geographic sources were already being maintained within laboratories at Kansas State University, Manhattan, Kansas (Chen et al. 2015). Insects from these laboratory populations were tested in the same way using a discriminating dose bioassay, but these populations were denoted as laboratory colonies. This category of lab-reared insects therefore included the long-term laboratory cultures and any field populations that were collected but then reared for three or more generations before being used in tests. The distinction between laboratory and field insects was important for this study as gene frequencies can easily change from those in field populations due to sampling effects (a small number of insects with gene frequencies not representative of the field population) random drift of frequency over time, and lack of selection pressure or fitness costs associated with resistance genes.

### **FAO Discriminating Dose Bioassay**

Presence and frequency of phosphine resistance in *T. castaneum* populations was determined using a discriminating dose bioassay described in Food and Agriculture Organization



of the United Nations (FAO) Method No. 16 (Food and Agriculture Organization 1975). All handling and treatment of insects in the bioassay was conducted at 25°C and 70% relative humidity. Insects were introduced to this environment for at least twenty-four hours before fumigation commenced. The number of insects that were used varied based on the size of the samples obtained from each populations, but three replications using separate fumigation jars were always used.

Twenty or more beetles were assayed for each replication of a given population and were placed in to 4 dram glass shell vials with a modified cap for ventilation. Vials contained approximately 500 mg of rearing medium as food for the beetles that lasted throughout the bioassay. Fumigation chambers were gas tight 3.8 L glass jars as described in Opit et al. (2012). Exposure vials within unique fumigation chambers were considered one replicate, and three replications for each population were subjected to the assay. Single replicates from multiple *T. castaneum* populations were assayed in one jar at the same time in many cases.

Unlike FAO method No. 16, phosphine gas was not generated using tablets (Food and Agriculture Organization 1975), but cylinderized phosphine at 10,000ppm was used to ensure accuracy and efficiency. This highly concentrated gas was moved from the cylinder in to gas tight CEL Scientific Tedlar® PVF film bags (CEL Scientific Corporation, Cerritos, CA), where it could be transferred with ease to many different fumigation chambers. Using a Hamilton® 25 mL, Model 1025 TLL gas tight syringe, 15.5 mL of 1% phosphine was added to fumigation chambers, after an equal amount of air was removed, to generate approximately 30 ppm of phosphine. This volume of gas was used to correct for the small amount that is generally lost to leakage. Directly after gas was introduced to these chambers the gas was mixed by pulling out the mixture and reinjecting. The concentration of the gas was then confirmed using a GC-FPD.

Fumigation lasted for twenty hours before jars were opened within a fume hood to allow for gas to leave the vials. Directly after fumigation began and before the jars were opened, the concentration within the jars was assessed. Using the start and finish concentration of phosphine, an average level of phosphine over the 20h period was obtained using quantitative GC (see below). All replicates that had averages outside of 25-35 ppm were thrown out as the discriminating dose was possibly significantly different than the target level. Insects were then kept in a growth chamber at the same temperature and relative humidity for fourteen days before mortality was assessed. For all bioassay replicates, the percent resistance was determined as the number of insects that were alive after a twenty hour fumigation at approximately 30 ppm, and fourteen days to allow for recovery, divided by number of insects tested and multiplied by 100. Percent resistance values were averaged across reps for reporting the results; replicates for which PH3 concentrations +/- 5 ppm or greater of the target 30 ppm were discarded from analyses. For every replication that was completed, a positive, susceptible control population (USDA- Center for Grain and Animal Health Research, Manhattan, KS) was included to be sure that the 30 ppm discriminating dose would kill all susceptible insects.

### **Dose Response Bioassay**

A dose response experiment was conducted to assess the levels of resistance that were present within both resistant and susceptible populations. For this bioassay, three geographically unique populations were used as representatives of three possible classes of resistance: no resistance or susceptible, weak resistance, and strong resistance. The susceptible population (USDA) was used so that resistant factors could be determined and compared to the other two possible resistance levels. For these two levels, one population was used that had a resistance frequency under 50% (Abilene2) which could possibly be a weak type resistance, and a strong

resistance was hypothesized to be present in individuals from a population, in this case from Red Level, AL, at 100% resistance. The methods used were similar to those of Opit et al. (2012), but this dose response used a twenty hour fumigation exposure instead of a 72 hr exposure used by Opit. Other differences included a seven day recovery period instead of fourteen days, as previous unpublished work showed no difference, and where possible, 50 beetles were used per replication. For each population a variety of increasing doses were used to generate dose-mortality data for deriving predicted lethal concentrations, so-called LC values. For the USDA susceptible strain, doses of 1, 2, 4, and 8 ppm were targeted, 10, 30, and 50 ppm for Abilene2, and 250, 500, 750, and 1000 ppm were targeted for Red Level. Although there were specific targeted doses, each dose determined by quantitative GC analysis was used as a separate mortality data point, with one targeted dose netting three different actual doses with associated mortality for use in the regression. Estimated LC values from a Probit regression analysis were determined using PROC PROBIT from SAS version 9 and plotted using log of phosphine concentration as the independent variable.

### **Comparison of Laboratory and Field Samples**

Populations chosen for comparing current resistance frequencies of field populations to laboratory colonies started several years in the past from the same field location were from those previously studied by Chen et al. (2015), who used seven laboratory culture collected a full three years (an estimated minimum of 18 generations) before assessing for phosphine resistance. Two populations in north-central Kansas from the original seven populations analyzed by Chen et al. (2015) were resampled from grain storage bins at or nearby the original collection sites. These two populations were analyzed using the FAO discriminating dose bioassay as described above.

## GC Analysis

Phosphine concentrations within the fumigation chambers were determined via quantitative gas chromatography (GC) using a Shimadzu GC-17A (Shimadzu, Kyoto, Japan) instrument equipped with a GS-Q column (30 m long  $\times$  0.53 mm i.d., 0.25  $\mu$ m 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  $\mu$ L, gas tight syringe. Operating conditions were as follows: injector temperature of 200 °C, detector temperature of 200 °C, and oven temperature isothermal at 150 °C. The injector was set in the split-less mode and the column flow rate was 4 ml/min. Ultra high purity helium was used as the carrier gas (Linweld, Lincoln, NE). For each replication in each experiment, three samples were taken to ameliorate experimental error. A phosphine standard of 200 ppm was generated by dilution of 1% phosphine with air in to a CEL Scientific Tedlar® PVF film bag. An external standard curve was generated with three injections of standard gas at volumes of 25, 20, 15, 10, and 5  $\mu$ l. The 15  $\mu$ l volume was designated 200 ppm, with subsequent injections at 5  $\mu$ l increments greater and less than 15  $\mu$ l representing an increase or decrease. The areas under the detector response curves for these injections were used to generate a standard curve with the linear equation of  $y = ax + b$ , for which  $y$  is the GC detector response,  $b$  is the  $y$ -intercept,  $a$  is the slope, and  $x$  is the  $\text{PH}_3$  concentration to solve for in experiment injections of 15  $\mu$ l.

## Results

### Resistance in North America

The phosphine resistance for each of 25 separate populations of *T. castaneum* across much of the United States and Canada are reported in (Table 2.1, Figure 2.1). Within the United States, 21 samples were assessed from a total of nine different states and one sample came from each for four Canadian provinces (Table 2.1, Figure 2.1). Thirteen of the populations had no resistant insects detected while some frequency of resistance was measured in the remaining twelve populations. Three of the six Kansas populations had insects determined to be resistant to phosphine. Minneapolis, KS had the highest frequency with 93%, while Abilene and Manhattan showed lower numbers of resistant insects with 41% and 15% respectively. Samples from Junction City, Kansas City, and Hudson had no beetles resistant to phosphine (Table 2.1). Of the five populations sampled from California (Table 2.1), only Goshen and Princeton had resistance, at 52% and 2%, respectively. Arbuckle, Davis, and Williams all showed no resistance to phosphine. The one population from the state of Texas showed similar levels of resistance to that of Princeton, CA, as Victoria, TX only showed 2% resistance when assayed (Table 2.1).

Populations from the State of Alabama and Florida both showed high frequencies of phosphine resistance (Table 2.1). Of the three populations assayed from Alabama, two populations showed resistance well above 90%. Both Ozark and Red Level populations showed high frequencies of resistance, and both were located in in the same geographic region of southeastern Alabama (Figure 2.1). However, Uniontown was located much farther to the west when compared to the first two sampled populations, and only exhibited 31% resistance (Figure 2.1; Table 2.1). The only sample population from the State of Florida was located very close to

the two highly resistant Alabama populations, and also had a high frequency of phosphine resistance at 88% (Figure 2.1; Table 2.1).

Many of the other populations assayed showed little to no resistance. Of the two populations from Georgia, one population had 22% resistance and the other was susceptible (Table 2.1). These populations were not very close to the more highly resistant Florida and Alabama populations (Figure 2.1). The only population from Arkansas showed 20% resistance, but the single populations from Missouri and Oklahoma were susceptible (Table 2.1). The population from Oklahoma was located near an area historically showing levels of resistance in this beetle (Figure 2.1) (Opit et al. 2012). Of all the populations of *T. castaneum* assayed from Canada, no population was found to have any sampled beetles resistant to phosphine.

Two of the populations from Kansas, Abilene and Minneapolis, were populations that had also been collected four years prior (Table 2.1; Figure 2.2) (Chen et al. 2015). When comparing the percent resistance of these two sample dates little difference was observed. Abilene<sup>2</sup> had 41% of sampled insects resistant when collected and assayed in 2014, compared to 39% resistant when collected in 2010 and assayed in 2013 (Table 2.1). Minneapolis showed similar results at 93% resistant when collected and assayed in 2014, compared to 89% resistant when collected in 2010 and assayed in 2013 (Table 2.1).

### **Analysis of Resistance Levels.**

Results of dose-mortality studies are reported in Table 2.2 and Fig.3. The lab-susceptible, Abilene and Red Level populations had mortality data that fell into three groupings with regard to phosphine concentrations to yield mortality. Calculation of resistance ratios based on the estimated LC<sub>50</sub> values suggests that Abilene beetles are several-fold more resistant to

phosphine than the susceptible strain, and that the Red Level population was about two orders of magnitude higher at 127.1-fold resistance.

## Discussion

Nearly half of the populations of *T. castaneum* studied in this project had some frequency of individuals scored by the FAO assay as being resistant to phosphine. These resistance frequencies varied greatly, with both high and low resistance frequencies occurring in the same broad geographical area. Although a lower percentage of resistant populations were found when compared to the survey of Oklahoma in 2012 (Opit et al. 2012), 48% vs. 89%, there were still a large number of populations assayed in this study with high resistance frequency. Also, the geographic extent of this study within the United States is only surpassed by that of Champ and Dyte (1976), who found approximately 10% of the populations sampled to be resistant with less than 10% resistance frequency in any of the populations. From the present study it is clear that phosphine resistance in *T. castaneum* occurs in many areas that store grain.

The two main regions that were previously assayed in 1989 and 1990, the southeastern states of Florida, Alabama, and Georgia, and states around Kansas, considered to be the midwestern states including and near Kansas, have shown a drastic rise in resistance frequencies in both 2012 and now in 2015 (Zettler et al. 1989; Zettler and Cuperus 1990; Opit et al. 2012). Populations with resistance frequencies as high as 93% occurred in Kansas, and 100, 98, and 88% in the collections from Alabama and Florida. These values suggest that resistance frequency has in fact increased over the years in those regions of the US, due likely to the large selection pressure exerted through high phosphine use. In the two cases for which a site was revisited in 2014 from a previous collection in 2010 (assayed in 2013), resistance levels were almost identical, if not higher. The population that was recorded as 89% resistant in 2013 was found to have a resistance level of 93% when recollected and assayed in 2014. However, the 2010 population was not assayed for phosphine resistance until 2013, but similar results were found in a less resistant population. This result on retention of resistance over time when compared to



laboratory colonization suggests two assumptions: resistance in the wild population is holding steady at a high level of resistance when compared to historic samplings (Chen et al. 2015), and populations without pressure from phosphine (three years) during laboratory culture are not rapidly losing their resistance phenotype. Thus it is very likely that phosphine resistance may not randomly disappear over a period of time due to either normal insect population genetics (e.g., immigration, emigration, drift) or human-caused impacts from pesticide use to storage of different crops over time.

Many populations studied here also showed a total lack of resistant beetles, which suggest that resistance alleles may be absent or at very low frequency in those areas. For California, although three out of the five populations showed no resistance, there were at least some levels of resistance found in both the northern and southern grain storage areas of the state. Canada has a more extreme situation compared to California because all four populations sampled had no evidence of resistance to phosphine. Lack of resistance in these locations suggest that genes for resistance are absent or at very low frequencies in these populations. For Canada we assume selection pressure for resistance is low, since many grain storages in Canada receive little to no phosphine fumigation in any given year (personal correspondence with Canadian Grain Commission). Gene flow, or migration of resistant *T. castaneum*, into regions can be explained by the occurrence of phosphine resistance at a site. The California populations are interesting for gene flow hypotheses, as the Goshen population is located far to the south (approx. 500 km) of the other four sampled. Lack of resistance in the northern California populations could be due to lack of key genes, with no infusion of resistance genes from the south, whether from restricted grain trade or limited *T. castaneum* dispersal over that distance. For Canada it is known that phosphine use is rare, and limited grain trade is assumed between the

two countries at those border locations. However, if *T. castaneum* are overwintering in high numbers in these areas, resistance is likely to develop considering pressure of phosphine use, if there is gene flow from resistant populations. For other areas that show susceptibility but are near highly resistant strains, it is likely that these are a result of resistant genes not moving in to those specific locations yet, either through grain movement or insect flight. These areas still have high phosphine use, with many locations that had susceptible beetles confirming the annual use of phosphine as the primary means of control. These susceptible populations near resistant populations were also seen in the other surveys of resistance in the United States (Zettler et al. 1989; Zettler and Cuperus 1990; Opit et al. 2012).

Resistant populations of *T. castaneum* studied here were represented by both weak and strong resistant phenotypes. Earlier work by Collins et al. (2002) with phosphine resistant populations of *R. dominica* in Australia found that there can be a large gap in the estimated concentrations needed to control weak and strongly resistant populations. Here it was found that the weak-resistant population from Abilene, KS showed only four times the resistance according to the LC<sub>50</sub> of a lab-susceptible strain, whereas the strongly resistant population for Red Level, AL was over 100 times resistant compared to the lab-susceptible strain. The dose response experiment performed here never achieved 100% mortality in the Red Level, AL strain even at doses around 1000 ppm. As also predicted by Opit et al. (2012), it is likely that these strongly resistant strains cannot be controlled with phosphine use any longer. Although work from this study and research by Opit et al. (2012) assessed the levels of resistance, this study looked at levels at shortened exposure times of only twenty hours. It could be possible that a shorter-than-recommended fumigation period is indicative of real world fumigations rather than an ideal three day steady phosphine level since average phosphine levels deplete much more quickly in

improperly sealed bins (Opit et al. 2012). Both methods give a different view, and this study gives the view of a much less efficient fumigation. From this data and Opit et al.'s (2012) conclusion, it is likely that strongly resistant populations cannot be controlled, but weakly resistant populations can be controlled with recommended fumigations that hold 200 ppm for at least three days (Phillips et al. 2012), or even with a 200 ppm fumigation for twenty hours. However, other life stages could be even more tolerant, as only adults were assayed. If these weak populations are still propagating resistance, it is likely that the issue of sealing bins is much worse than thought, or there could be some benefit to the fitness of resistant individuals which allows it to increase.

In other areas of the world such as Australia, monitoring programs have been administered to better understand phosphine resistance across all grain storage areas (Emery et al. 2011). These programs are important for countries that rely on phosphine to control stored product beetles, especially *T. castaneum*. For proper grain management, a program that is based off of resistance management techniques should likely be administered in the United States, as this study shows that resistance is possibly widespread. The answer to controlling phosphine resistance lies in proper resistance management. The first step should be in a proper monitoring program, which contains a commercial rapid bioassay that allows for knowledge of presence or absence of resistance within one day or less of the finding of a *T. castaneum* infestation (Emery et al. 2011). Next, proper sealing of bins needs to be accomplished before any more fumigations are administered (Emery et al. 2011). Not only is fumigating an unsealed bin possibly a waste of money if the strains are strongly resistant, but fumigating without properly sealing first is likely selecting for resistance. Another possible alternative for bins that may not allow proper sealing is a constant flow phosphine system (Anderson 1989). This allows for phosphine to stay at a steady

level for extended periods of time, unlike pellets placed in to a leaky bin (Anderson 1989).

Lastly, if resistant strains are found that are too resistant to treat, a proper alternative should be used such as ProFume® (Drinkall et al. 2012). This fumigant has a different mode of action than phosphine, so beetles resistant to phosphine likely shouldn't garner resistance to it as well.

Now that phosphine resistance in *T. castaneum* is known to occur across much of the United States, more research needs to be performed to augment these findings. Research has investigated so-called quick tests to shorten the time needed to assay for resistance, but more effort needs to be allocated in assessing resistance altogether (Reichmuth 1991; Chen et al. 2015). With the repeated documentation of resistance, grain managers will start to understand that phosphine treatment may not be the best option for every fumigation. Add this in with a rapid measure to identify resistance, and grain managers could possibly be properly equipped for resistance management. With these recommendations and more, it will be possible to save phosphine from becoming one of the treatments of the past, like many pesticides have become.

## References

- Anderson, I. 1989.** Pumps deliver fatal blow to grain pests. *New Scientist* 122: 38.
- 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. *Journal of Stored Prod. Res.* 40: 241–249.
- Borah, B. and B. S. Chahal. 1979.** Development of resistance in *Trogoderma granarium* Everts to phosphine in the Punjab. *FAO Plant Prot. Bull.* 27: 77- 80.
- 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.
- 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.
- Collins, P. J., G. J. Darglish, 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.
- 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. Kansas Entomol. Soc.* 59: 620-627.
- Drinkall M.J., V. Zuffagnini, L. Suss, and D.P. Locatelli. 2002.** Efficacy of sulfuryl fluoride on stored product insects in a semolina mill trial in Italy, in *Advances in Stored Product Protection*, pp. 884-887. *In Proceedings of the 8th International Working Conference on Stored Product Protection*, 22-26 July 2002, York, UK. CAB International, Wallingford, United Kingdom.

- Emery, R. N., M. K. Nayak, and J. C. Holloway. 2011.** Lessons learned from phosphine resistance monitoring in Australia. *Stewart Posthar.* 7: 1-8.
- Flinn, P. W., D. W. Hagstrum, C. Reed, and T. W. Phillips. 2003.** United States Department of Agriculture-Agricultural Research Service stored-grain area wide integrated pest management program. *Pest Man. Sci.* 59: 614-618.
- 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.
- Hagstrum, D. W., C. Reed, and P. Kenkel. 1999.** Management of stored wheat insect pests in the USA *Integrated Pest Manag. Rev.* 4: 127-142.
- Hagstrum D. W., T. W. Phillips, and G.W. Cuperus. 2012.** *Stored Product Protection.* Kansas State University. Manhattan, KS. KSRE Publ; S-156; 2012.
- Lorini, I., P. J. Collins, G. J. Darglish, M. K. Nayak, 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.
- 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.
- Phillips, T., E. Thoms, J. Demark, and S. Walse. 2012.** Fumigation, chapter 14. *In* *Stored Product Protection.* K-State Research and Extension publication S156.
- Reichmuth C. 1991.** A quick test to determine phosphine resistance in stored-products research. *GASGA Newsletter* 15: 14-15.

- Sayaboc, P. D., A.J.G. Gibe, and F. M. Caliboso. 1998.** Resistance of *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) to phosphine in the Philippines. Philipp. Entomol. 12: 91-95.
- Tyler, P. S., R. W. D. Taylor and D. P. Flees. 1983.** Insect resistance to phosphine fumigation in food warehouses in Bangladesh. Int. Pest Control 25: 10-13.
- 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., 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.

**Table 2.1 Percentage of individuals found resistant to phosphine in North America populations of *Tribolium castaneum* using the FAO discriminating dose bioassay.**

Population	State/Prov. <sup>1</sup>	City <sup>2</sup>	GPS Coordinates (N, W) <sup>4</sup>	% Resistant (n)
1	AL	Uniontown*	32.445, -87.513	31 (59)
2		Red Level	31.407, -86.612	100 (40)
3		Ozark	31.459, -85.640	98 (40)
4	AR	Jonesboro*	35.842, -90.704	20 (80)
5	CA	Arbuckle	39.017, -122.057	0 (30)
6		Davis	38.544, -121.740	0 (60)
7		Goshen*	36.351, -119.420	52 (63)
8		Princeton*	39.403, -122.009	2 (60)
9		Williams	39.154, -122.149	0 (30)
10	FL	Walnut Hill*	30.885, -87.509	88 (80)
11	GA	Nashville	31.207, -83.250	22 (30)
12		Tifton*	31.450, -83.508	0 (60)
13	KS	Abilene <sup>3</sup>	38.917, -97.213	41 (30)
14		Hudson*	38.105, -98.660	0 (80)
15		Junction City	39.028, -96.831	0 (30)
16		Kansas City*	39.114, -94.627	0 (80)
17		Manhattan	39.183, -96.571	15 (30)
18		Minneapolis <sup>3</sup>	39.121, -97.706	93 (60)
19	MO	Excelsior Springs	39.339, -94.226	0 (60)
20	OK	Calumet*	35.601, -98.118	0 (60)
21	TX	Victoria	28.805, -97.003	2 (50)
22	MB	Winnipeg	49.899, -97.137	0 (30)
23	QC	St. Agathe	46.383, -71.409	0 (30)
24	SK	Saskatoon	52.133, -106.670	0 (60)
25	AB	Calgary	51.048, -114.070	0 (60)

<sup>1</sup>Postal codes for Canada provinces and USA states used for identification of collection sites.

<sup>2</sup>Populations denoted with (\*) were tested after being in the laboratory for > 3 generations.

<sup>3</sup>Populations from Abilene and Minneapolis are revisited sites from Chen et al. 2015.

<sup>4</sup>GPS coordinates are for cities, not exact locations of populations.



**Table 2.2 Probit analysis for mortality responses of adults from selected populations of *T.c.* to varying concentrations of phosphine during 20 hr exposure at 25 °C.**

Population	LC <sub>50</sub> (95% FL) ppm	LC <sub>90</sub> (95% FL) ppm	LC <sub>99</sub> (95% FL) ppm	$\chi^2$ (df)	RR <sub>50</sub>
USDA	5.17 (4.78-5.57)	8.03 (7.28-9.21)	11.49 (9.89-14.41)	4.2192 (6)	1.00
Abilene	23.19 (17.69-28.86)	36.87 (29.52-56.93)	53.81 (39.89-111.28)	3.7472 (7)	4.49
Red Level	657.19 (605.87-709.49)	1431 (1242-1755)	2697 (2120-3863)	12.4629 (8)	127.11

Figure 2.1 Map of North America showing approximate geographic locations of the twenty-five populations of *Tribolium castaneum* analyzed for phosphine resistance. All locations and scale are approximate. Kansas populations included in a separate map due to close proximity of sites.

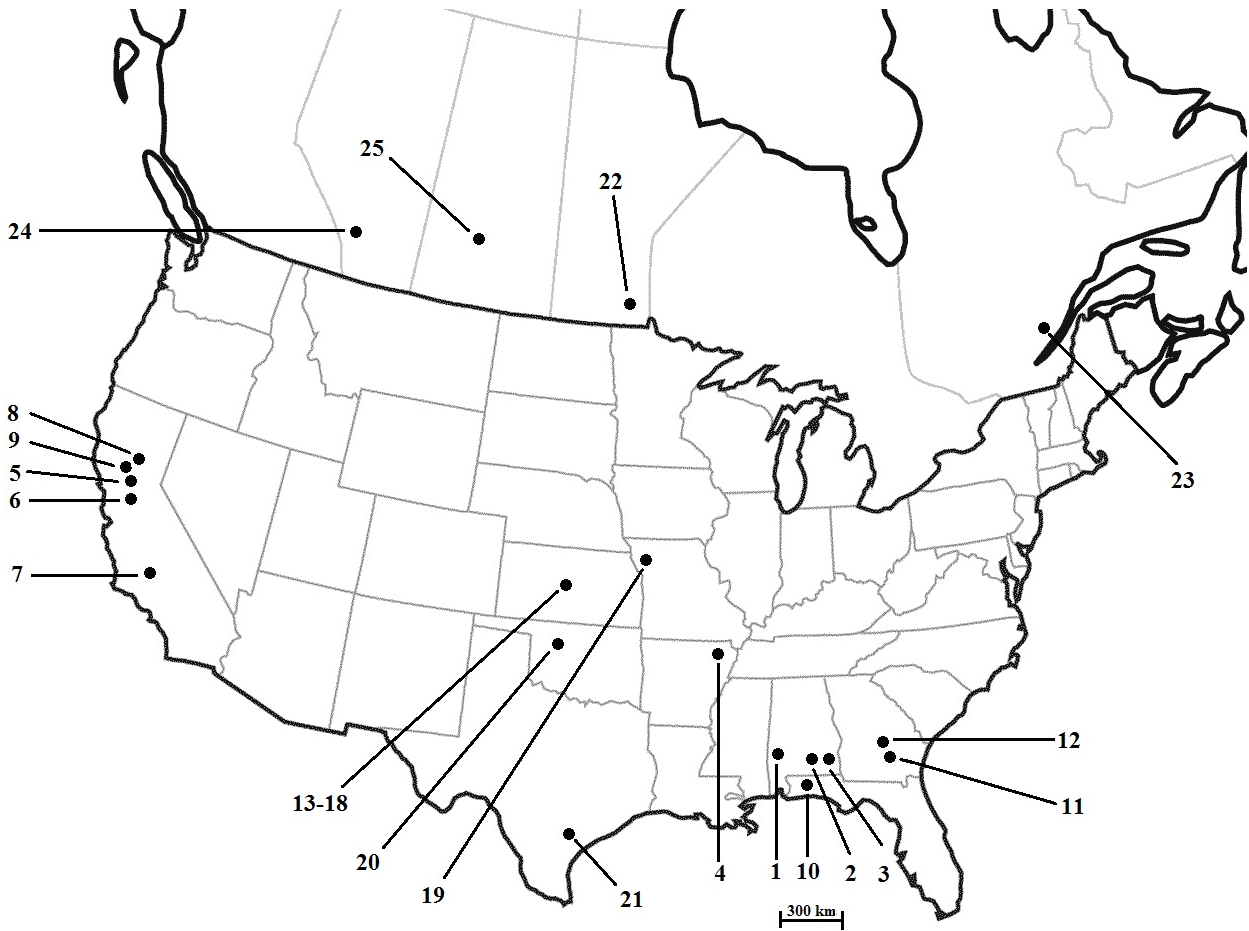
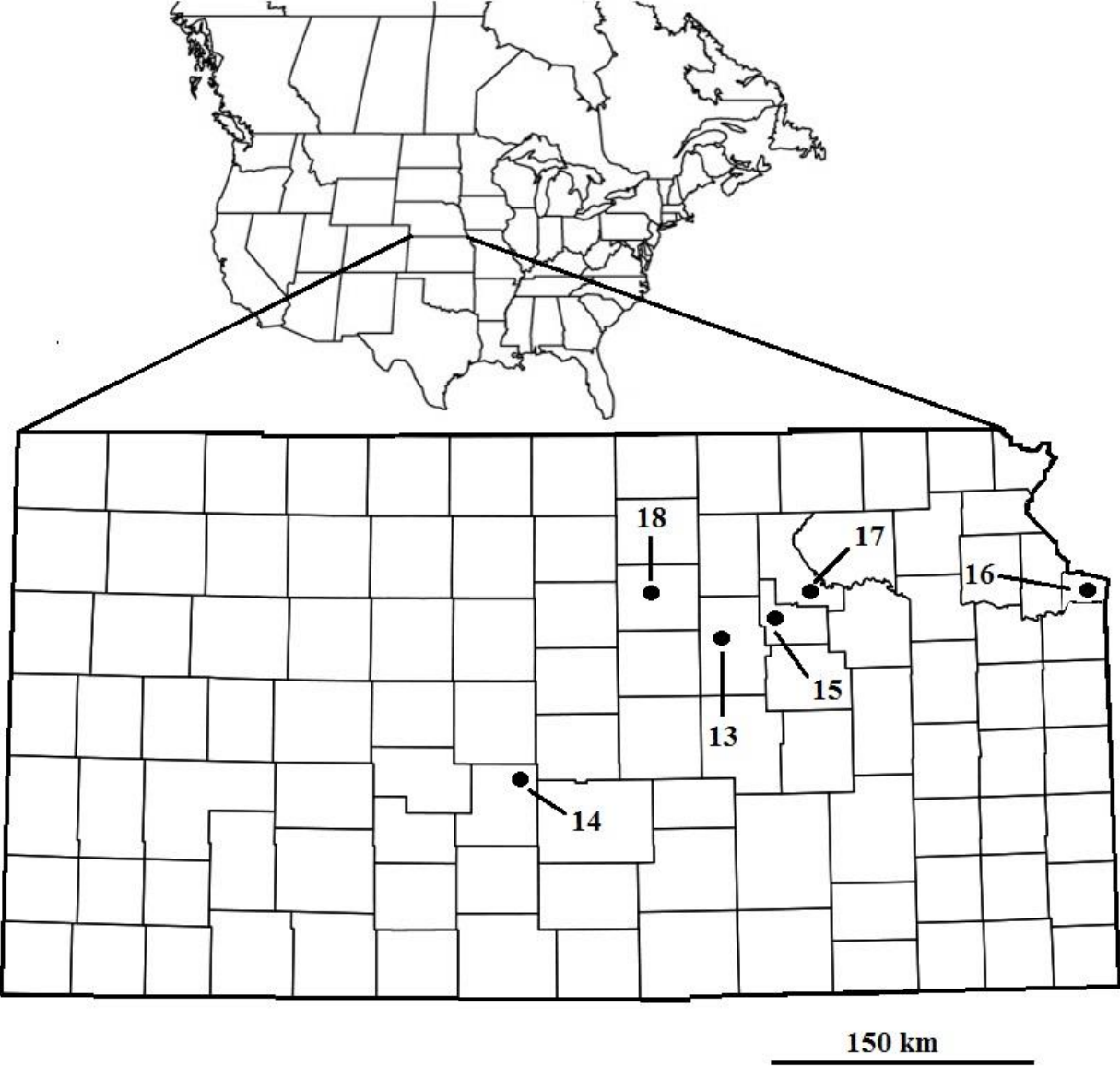
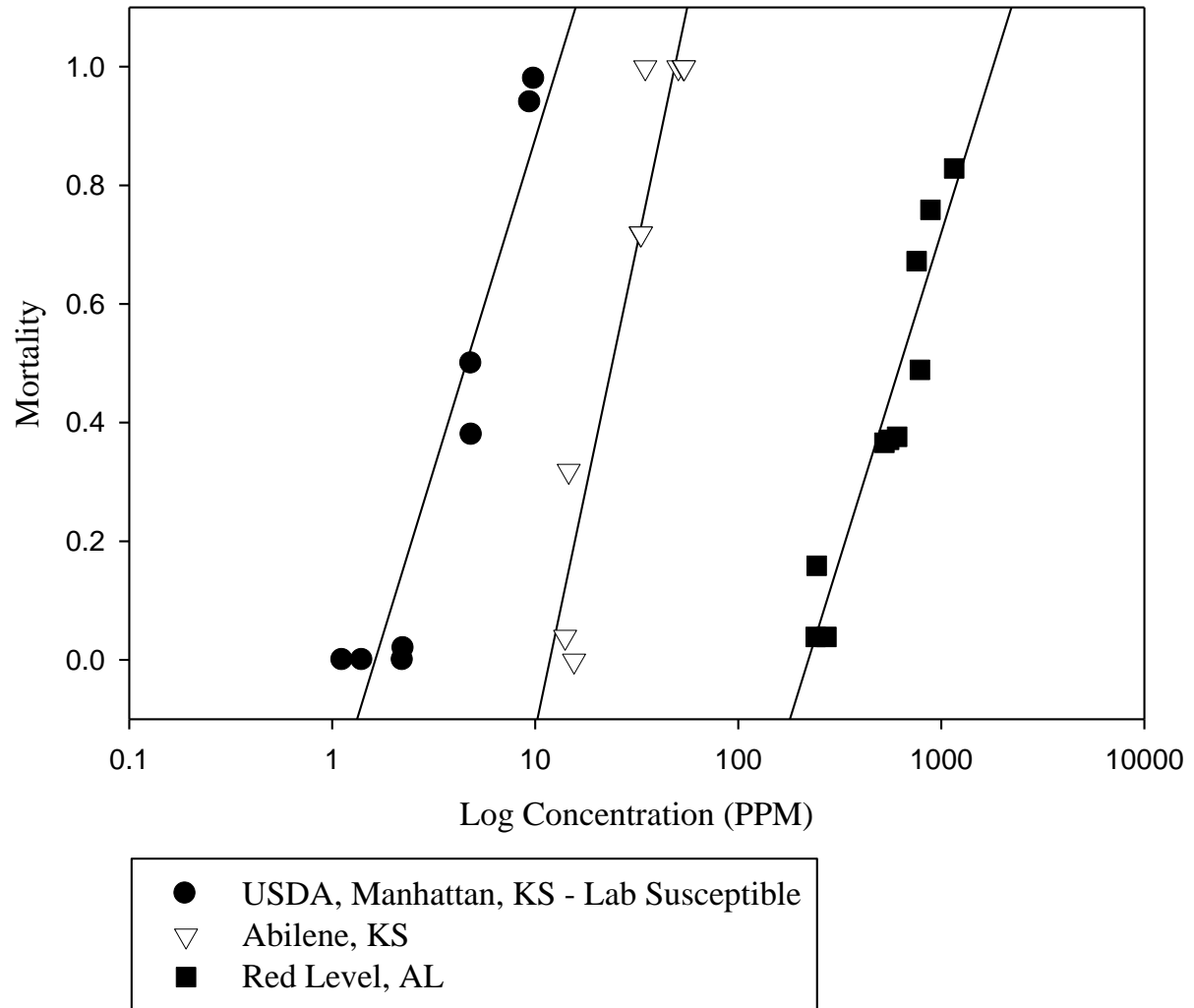


Figure 2.2 Map of Kansas showing approximate geographic locations of six populations of *Tribolium castaneum* analyzed for phosphine resistance. All locations and scale are approximate.



**Figure 2.3 Dose-response plots for populations subjected to Probit analyses and designated as having phenotypes of phosphine-susceptible, weak resistant and strong-resistant.**



**Chapter 3 - Evaluation and Refinement of Knockdown Bioassays to  
Assess Phosphine Resistance in the Red Flour Beetle, *Tribolium  
castaneum* (Herbst)**

## Abstract

Although phosphine resistance has been identified in *Tribolium castaneum*, proper methods to quickly identify resistance have not been commercially adopted. The purpose of this study was to assess the effectiveness of different types of quick tests based on adult knockdown time, and to identify ways to make quick tests more user-friendly. Using a set, easy to understand definition of knockdown and 10 beetles per replication in glass culture tubes gassed to 3000 ppm, bioassays determined that the  $KT_{50}$  is likely to be the most efficient parameter to determine resistance. The  $KT_{50}$  was determined to be more efficient because bioassays would be much shorter, with some populations such as Red Level, AL having a  $KT_{50}$  of 24 minutes and a  $KT_{100}$  of 194 minutes. Using the  $KT_{50}$ , a knockdown test should look to identify strongly resistant *T. castaneum* populations rather than susceptible vs. resistant populations. Because of the high variability among weakly resistant and susceptible insects, 7.89 minutes in a resistant vs. 10.05 minutes for a susceptible population, results are not clear when comparing the two. However, it was found that strongly resistant populations showed a clear difference when compared to susceptible and weak populations. With these findings in hand, augmenting commercially ready kits with updated methods could possibly lead to a quick test that grain managers can and will easily use. With the use of such a quick test, real-time resistance management decisions can be made to help combat the growing problem of phosphine resistance.

## Introduction

Stored grains are attacked by many types of invaders throughout periods of storage which generally last for one month to one year or more (Hagstrum et al. 2012). A variety of organisms and conditions deteriorate the quality and quantity of stored grains and finished products, which can lead to substantial economic impacts. One of the most cosmopolitan pests to cereal grains and milled grain products is the red flour beetle, *Tribolium castaneum* (Coleoptera: Tenebrionidae) (Cuperus et al. 1986). This pest is generally controlled through fumigation with hydrogen phosphide gas, also known as phosphine. However, within the last half century, it has been known that populations of this species can be resistant to phosphine. Resistance was first reported in the United States in 1976 (Champ and Dyte 1976), later confirmed by two different studies in the late 1980s-early 1990s, and one study in 2012 (Zettler et al. 1989; Zettler and Cuperus 1990; Opit et al. 2012) and further validated in Chapter 2 of this thesis. These studies not only confirmed the existence of resistance in this pest species, but they also showed a trend in the steady increase of both resistance prevalence and strength from 1976-2015.

Although resistance can be identified, there are problems that exist with the process of using a discriminating dose bioassay with this fumigant. The most commonly used test to diagnose phosphine resistance is FAO method number 16 (Food and Agriculture Organization 1975) and variations thereof. The FAO methods use a discriminating dose bioassay with concentrations included for seven different pest species, including *T. castaneum*. This bioassay requires a twenty hour fumigation at 30 ppm for *T. castaneum*, and then fourteen days post fumigation to allow for possible recovery or delayed mortality in the tested sample (Food and Agriculture Organization 1975). The discriminating dose, the concentration at which all susceptible beetles die, and above which resistant beetles survive, is typically derived from the estimated concentration needed to kill 99.9% beetles from a susceptible population. Although

the FAO bioassay is adequate to detect resistance in a given population, it requires key analytical instruments and careful scientific techniques that are not practical for grain managers or commercial fumigators to possess. These bioassays need to be conducted in specialized biology or chemistry laboratories. Impracticalities for commercial applications have led many to consider developing and using a simple one-day test that is practical and inexpensive compared to the FAO assay.

A common “quick test” for phosphine resistance is based on a biological phenomenon known as narcosis, which is assessed by observing and recording aspects of adult knockdown that usually does not cause death. When insects are exposed to phosphine at a high concentration (around 0.5 mg/l, 360 ppm, or higher) they will enter a narcotized state wherein the majority of the insects will become inactive and be active again if exposure times are not long (Winks 1984). Thus, resistant insects are expected to tolerate longer times under gas before knockdown, and susceptible insects should be knocked down at shorter times, all times being relative to the type of test among other variables. Bang and Telford (1966) found that as the concentration of phosphine was increased, the time to narcotization decreased. Reichmuth (1991; 1994) found that resistant *R. dominica* took up to 80 minutes longer to be narcotized than susceptible beetles of the same species, which he later attributed to a lowered uptake of phosphine in resistant beetles. Waterford and Winks (1994) then confirmed that through selective breeding of a susceptible population, narcotization could be delayed, which correlates with increased resistance itself. The discovery of this narcosis phenomenon and its relationship with phosphine susceptibility or resistance has been an exciting avenue for developing accurate and cost-effective phosphine resistance bioassays.



Many bioassays have been created that use the time to knockdown of samples from different populations as the main criterion (Reichmuth 1991; Bell et al. 1994; Cao and Wang 2000; Steuerwald et al. 2006). Of these rapid bioassays there is much overlap, but also many differences. The main differences lie in the time required for a given percentage of the population, whether 50% or 100%, that is being knocked down ( $KT_{100}$  or  $KT_{50}$ ), the criteria for what exactly defines insects in the bioassays as being knocked down, and how commercially marketable each test was made to be. Some of these bioassays were created as a method for laboratories, and most notably, Steuerwald's et al.'s (2006) bioassay was created to be used at the location of grain storage itself. Each of these bioassays were capable of accessing phosphine resistance through the narcotization behavior within 30 minutes of the beginning of the bioassays, thus yielding a useful result in much less time than the standard FAO discriminating dose bioassays. Also, these tests correlated well when compared with dose response bioassays (Reichmuth 1991; Bell et al. 1994; Cao and Wang 2000; Steuerwald et al. 2006).

These rapid bioassays have shown that a more efficient test is possible, but even though these studies were successful, there still has yet to be an adoption of such a test in real world situations. Many of these problems exist because the bioassays were not created for commercialization. These problems include the lack of explanation of what the knockdown behavior actually means to a layman, sophisticated methods meant for use in laboratory work, and use of the  $KT_{100}$  rather than a more efficient  $KT_{50}$  that will likely harbor less variation. For the added benefit of the commercialized methods, this study reported below focused on improving work previously done by Steuerwald et al. (2006), which lead one company to develop a prototype quick test using knockdown times (Degesch America, [www.degeschamerica.com](http://www.degeschamerica.com)). The overall goal of this study is 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. The first objective was to assess the ability of the FAO discriminating dose bioassay to explain the time to knockdown in three different quick test bioassays:  $KT_{100}$ ,  $KT_{50}$ , and knockdown of single individuals. A subsequent objective was to assess the importance of movement or disturbance, such as that caused by one beetle on another in a group, in the quick test environments.

## **Materials and Methods**

### ***Tribolium castaneum* Population Rearing and Selection**

For all quick test bioassays performed, insects were taken from eleven populations studied in Chapter 2 that were reared using a standard protocol. One additional population named “Thailand” (Table 3.1) was not studied in Chapter 2 and was a long-term laboratory colony received from colleagues in Canada who made the initial collection in Thailand. Beetles were reared in Ball® 473 ml Wide Mouth Mason Jars, and a metal screen combined with filter paper was used instead of the supplied metal lid insert. These beetles were reared on organic golden buffalo flour supplemented with 5% brewer’s yeast (95:5). Colonies were kept within growth chambers set at 28°C and 65% relative humidity with a photoperiod of 16hrs light and 8hrs dark. Selection of colonies chosen for quick test assessment was based on two key factors: level of resistance and adequate numbers of beetles in culture at the time. The main focus was based on the resistance levels of each population, more specifically populations from the three groups of resistance were prioritized: susceptible, weak, and strong resistance. There happened to be populations that fit in to these three categories, however, many of the resistance levels in between were either non-existent, or some of those populations had small numbers when sampled and could not be used for this study.

### **The Quick Test Technique**

For the first part of this chapters’ studies it was determined that a model for a commercial quick test should be chosen. Steuerwald et al. (2006) reported a simple knockdown assay in which beetles were placed in a large plastic syringe to which a high concentration of phosphine was introduced from small gas-tight vessel that contained gas generated from small tablets of magnesium phosphide. Analyses were performed with the purpose of augmenting these

techniques so that the kit created in this study could possibly be used along with the methods that would be determined as the most efficient. Concurrent with the instructions of the kit, it was determined that 3000 ppm was an acceptable level of phosphine that wasn't too high or too low for use, and could easily be reached using the magnesium phosphide pellets found within the commercial kit (Steuerwald et al. 2006). However, it was determined that all data would be collected using a different setup than the Steuerwald et al. 2006 kit to ensure that data were accurate and repeatable.

For all quick test trials in this chapter, a standard procedure was used for the environment the insects were tested in, and for the methods to introduce gas. PYREX® 55mL Screw Cap Culture Tubes with PTFE Lined Phenolic Caps, 25x150mm, were used as fumigation chambers with a Fisherbrand™ Turnover Septum Stopper used to make the tubes gas tight and allow for introduction of phosphine. Insects were added in to these vials and were not introduced with a food substance. Using a Hamilton® 25 mL, Model 1025 TLL gas tight Syringe, 16.5 mL of 1% phosphine was added to the glass tubes 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. Analysis of the fumigation tubes once gas was added was done by a GC-FPD (see below).

Within the available literature there seems to be either ambiguity in how a particular test determined if a beetle is knocked down, or a chosen method of scoring knockdown was ambiguous itself. Many preliminary trials were performed to see which types of knockdown assessment would generate the least error. Because of the expectation of variation, scoring methods such as those that used descriptions such as “hampered insects”, “inactivity”, or “the insect’s inability to walk” were avoided (Reichmuth 1991; Bell et al. 1994; Steuerwald et al.

2006). A more definitive way of scoring knockdown was employed, which is to record the time at which there is a persistent and complete lack of movement by a treated insect. This means that if a beetle is on its back, but its legs are still twitching, it would not be scored as knocked down. Although inactivity and hindrance alone were excluded from the scoring method, it is still likely that these are the beginning stages of the narcosis phenomenon and are viable ways to determine if a beetle is beginning to have an increased tolerance to phosphine. However, it was felt that such scoring methods would not be adequately unambiguous for a commercialized test, as not every untrained grain manager using this test will agree with what inactivity means.

Included with the definition of complete lack of movement that was used for this study, it was determined that when a replication contained multiple beetles, some beetles would go from completely knocked down to active again over the course of one trial. Because of this, studies were modified by adding in a 30-second count when the last beetle of question becomes completely motionless. For replications with multiple beetles, this helped to control for possible error that could occur when one beetle is left to be knocked down, and as soon as it becomes motionless, a different beetle within the replication moves again. This switching between moving and not moving creates uncertainty about whether the test insect is actually completely “narcotized” at that point. The 30 second count was applied to all beetles within a replication, and if movement was observed during this count, the count was continued until all beetles of interest (the single beetle, 50%, or 100% of a group) were again motionless for more than 30 seconds.

### **Evaluating Knockdown Times**

To better understand how efficient different types of quick tests were, three main methods were assessed: the time to knockdown of single individuals from a population, the time

to knockdown of 50% (KT<sub>50</sub>) of a sample of ten insects from a population, and the time to knockdown of 100% (KT<sub>100</sub>) of a sample of ten insects from a population. Both the time to 50% and 100% knockdown of a population are the traditional methods used in knockdown tests, however single insect knockdown is rarely used in actual tests. Single insect knockdown was included mainly for comparison, as it was decided to test efficiency of the test rather than assume any group or sub-group gave similar or more accurate estimates of resistance.

Single insect trials were tested by using one insect per replication (n=10) for the 12 different populations. Time to knockdown was determined using methods described above with a complete lack of movement for at least 30 seconds scoring as knocked down. For both KT<sub>50</sub> and KT<sub>100</sub> trials 10 insects were used per replication (n=5) with a complete lack of movement for at least thirty seconds for either 50% or 100% of the beetles scoring as knocked down. When a trial was started there were ten beetles in the replication, and the time to knockdown for both 5/10 and 10/10 of those beetles were recorded for each population, yielding one replication for both KT<sub>50</sub> and KT<sub>100</sub>. However, since the setup was different for single insect trials, those were done independently.

### **Effect of Beetle Stimulus on Knockdown Time**

To better understand the reason for shorter knockdown times in single insect trials a disturbance was applied to the glass knockdown chamber to stimulate the test insect as an experimental treatment. Single insect trials were completed for two separate populations, a susceptible and resistant population. In these trials, the knockdown tubes received one of two different treatments; either the tubes were not moved at all during the full duration of the knockdown test, or every minute tubes were disturbed by rolling approximately 360° in one direction and then 360° back to their starting place. Preliminary studies showed that if the glass

tubes were moved around in some fashion that disturbed or stimulated the beetle during a trial, the time to knockdown of that beetle would increase compared to unstimulated beetles. Trials were performed to determine if this phenomenon was consistent in both susceptible and resistant populations, and if the stimulation phenomenon would create a statistically significant difference in knockdown times. For the susceptible (n=40) and the resistant (n=20) beetles, an equal number of trials were run with stimulus added and no stimulus. These two populations were then compared independently using an Unpaired T-Test with the R (R Development Core Team 2013) statistical program. A p-value lower than 0.05 indicated that stimulus caused a significant increase in the time to knockdown, possibly affecting knockdown tests. Although single insects were used in these trials, earlier trials saw similar results in KT<sub>100</sub> studies, as some populations were not knocked down within four hours with the added stimulus.

### **GC Analysis**

Phosphine concentrations within the fumigation chambers were determined via quantitative gas chromatography (GC) using a Shimadzu GC-17A (Shimadzu, Kyoto, Japan) instrument 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, gas tight syringe. Operating conditions were as follows: injector temperature of 200 °C, detector temperature of 200 °C, and oven temperature isothermal at 150 °C. The injector was set in the splitless mode and the column flow rate was 4 ml/min. Ultra high purity helium was used as the carrier gas (Linweld, Lincoln, NE). For each replication in each experiment, three samples were taken to ameliorate experimental error. A phosphine standard of 200 ppm was generated by dilution of 1% phosphine with air in to a CEL Scientific Tedlar® PVF film bag. An external

standard curve was generated with three injections of standard gas at volumes of 25, 20, 15, 10, and 5  $\mu\text{l}$ . The 15  $\mu\text{l}$  volume was designated 200 ppm, with subsequent injections at 5  $\mu\text{l}$  increments greater and less than 15  $\mu\text{l}$  representing an increase or decrease. The areas under the detector response curves for these injections were used to generate a standard curve with the linear equation of  $y = ax + b$ , for which  $y$  is the GC detector response,  $b$  is the y-intercept,  $a$  is the slope, and  $x$  is the  $\text{PH}_3$  concentration to solve for in experiment injections of 15  $\mu\text{l}$ .

### **Data Analysis**

For each population tests for single insect trials,  $\text{KT}_{50}$ , and  $\text{KT}_{100}$  were completed and compared to FAO resistance frequencies using a regression analysis. The percent resistance as determined by the FAO discriminating dose bioassay was used as the independent variable and time to knockdown for each of the three different knockdown assays was used as the dependent variable. These tests were ran using a regression analysis function in the R (R Development Core Team 2013) statistical program, and  $r^2$  values were compared to determine the efficiency of each type of knockdown procedure. One-way ANOVAs with post-hoc Tukey's HSD tests were used to identify significant variation in raw sample data between levels of the treatments within single populations. Two-way ANOVAS with post-hoc Tukey's HSD tests were used to identify significant variation in raw sample data between levels of the treatments and the interaction of different treatments and populations. All ANOVAS were performed using the R (R Development Core Team 2013) statistical program.



## Results

### Assessment of Quick Test Techniques.

For a total of twelve different populations of *T. castaneum*, three different quick test treatments were assessed (Table 3.1). For the quick test using single beetles (n=10), knockdown times ranged from 8.46-52.70 minutes (Table 3.1). This quick test technique showed that the percent resistance according to the FAO discriminating dose bioassay explained time-to-knockdown reasonably well, exhibiting an  $r^2$  value of 0.652 ( $p < 0.001$ ) (Figure 3.1). The knockdown test using  $KT_{50}$  (n=5) had knock down times ranging from 5.19-24.33 minutes (Table 3.1). The ability of percent resistance to explain time-to-knockdown according to the  $KT_{50}$  was estimated with an  $r^2$  value of 0.843 ( $p < 0.001$ ) (Figure 3.2). Of the three different types of quick tests assayed, the time-to-knockdown according to the  $KT_{100}$  was explained best with an estimated  $r^2$  value of 0.873 ( $p < 0.001$ ) (Figure 3.3). Time-to-knockdown from the  $KT_{100}$  ranged from 9.13-194.13 minutes (Table 3.1).

Analysis of variation between the quick test treatments among a population was also performed (Table 3.1). For every population, there was a significant difference between the different tests with p-values ranging from  $p = 0.002$  to  $p < 0.001$  (Table 3.1). In this analysis, the specific differences between each knockdown test were assayed using a post hoc analysis, with results showing that not all of the tests were statistically different for each population (Table 3.1). A trend among the populations emerged with the  $KT_{100}$  being statistically different from the other two tests in all but the USDA (USDA, Manhattan, KS) population (Table 3.1). It was found that the single knockdown test and  $KT_{100}$  were not statistically different, while the  $KT_{50}$  was different than both of the other tests (Table 3.1). For every other population except USDA, the single knockdown test and  $KT_{50}$  were not found to be statistically different (Table 3.1).

A further analysis was performed to determine the differences among the populations within a given KT type ( $F=92.952$  and  $p<0.001$ ) (3.1). For the single insect trials, all populations from USDA through Russell and including Mitchell were not statistically different from each other, and only Red Level, with an FAO frequency of 100%, was different than all other populations for this quick test type (Table 3.1). The  $KT_{50}$  test differed a bit more across populations because two of the susceptible populations (Arbuckle and Williams) were found to have statistically larger KT times than the three susceptible populations with the lowest KT (Table 3.1). Populations with a low frequency of resistance had statistically different KT's than all but one strong resistant population, but Red Level was once again different than all other populations (Table 3.1).  $KT_{100}$  results showed the best separations of populations by FAO resistance frequency, but these were not usefully sub-divided as most of the susceptible and weak populations were found to be not statistically different (excluding Russell vs. USDA). Of the strains with a high frequency of resistance, two were similar to each other (Mitchell and Red Level), and Minneapolis was only similar to Russell (Table 3.1).

### **Analysis of Stimulus during Knockdown Trials.**

The mean knockdown time for susceptible beetles without any stimulus applied to the assay vial was 7.43 (SE=0.31) (n=40) minutes, while beetles with a stimulus added took on average 8.81 (SE=0.31) (n=40) minutes to knockdown (Figure 3.4). For the resistant population, the mean knockdown time for beetles without stimulus was 13.03 (SE=1.16) (n=20) minutes, and 32.94 (SE=3.47) (n=20) minutes for beetles with the added stimulus (Figure 3.4).

## Discussion

Comparing knockdown tests to mortality based tests is not a novel idea (Reichmuth 1991; Bell et al. 1994; Waterford and Winks 1994; Cao and Wang 2000), however, this study compares the similarities in the FAO discriminating dose bioassay with three knockdown test methods. In addition, this study used twelve geographically different populations with a range of susceptibility to better explain the differences among resistance groups. Results from this study showed that within susceptible populations, as denoted by the FAO discriminating dose bioassay, a range of knock down times are present. The USDA strain is a true susceptible laboratory population, but there were five other susceptible field populations included that showed knockdown times more than doubling in the  $KT_{50}$  and  $KT_{100}$  tests. From this it can be assumed that if a discriminating time to knockdown is created, there must be many susceptible populations studied before this type of test could truly distinguish something resistant from strongly tolerant. Also, for both  $KT_{50}$  and  $KT_{100}$ , there were a few susceptible populations that took longer to knockdown than a weakly resistant population. This could mean that when looking at some susceptible populations and weakly resistant populations only, a clear picture is not present when using time-to-knockdown. Although these may not have been as clear, when moving to more highly resistant populations, knockdown time increased drastically. This means that it should be easy to identify possible non-controllable populations in the field with this method, rather than just looking at resistant vs. susceptible from an FAO-type assay.

As seen in other studies, the  $KT_{50}$  has been shown as an efficient way of scoring resistance in a population (Cao and Wang 2000; Waterford and Winks 1994). The data from this study supports the idea that the  $KT_{50}$  is an efficient knockdown test, and it is recommended that a commercial quick test use this metric. The research conducted in this study shows that using a very standard definition of knockdown was possible, rather than using an ambiguous definition.

If a useable commercial quick test is to be designed, a concise definition for knockdown needs to be implemented to ensure lack of confusion and increased accuracy.

Looking at knockdown data from susceptible and resistance *T. castaneum*, it was determined that an added physical stimulus during knockdown trials increased the time to knockdown of the beetles. This stimulus applied in this experiment simulated beetles being moved around, contacting each other, or falling over, and may be related to what they experience in groups with other beetles. The difference that was observed in a susceptible population was much smaller when compared to the resistant population. In the resistant population, the time-to-knockdown was close to three times higher when a stimulus was added. The stimulus driven knockdown time of the resistant population in single trials was similar to that of other populations with the same resistance level for the  $KT_{100}$ . With this study, it is clear that movement of bioassay environments could skew knockdown tests and possibly give bad data if not all replications were treated the same. In the same way, this knowledge is helpful because assays which beetles are allowed to continually crawl up a surface and fall, likely are not comparable to assays where insects are only allowed to move horizontally (Bell et al. 1994; Hori and Kasaishi 2005).

Results from this study suggest a number of recommendations for further development and use of Steuerwald et al.'s (2006) commercial quick test. First, implementation of a more easily understood definition of knockdown will decrease ambiguity in the test. The current definition uses the descriptor "active" following exposure to describe resistant insects and gives no more explanation (Steuerwald et al. 2006). Second, moving from a  $KT_{100}$  to a  $KT_{50}$  will increase and streamline the efficiency of the test overall due to the shorter time needed, as knockdown times will decrease but utility of the assay should not. Third, before implementation

of this test, discriminatory times for resistant beetles need to be based on more than one population. It has become evident that one highly susceptible population does not necessarily have the same time-to-knockdown as other susceptible populations. If the data from this study were used, especially following additional research on more populations, a more accurate discrimination range for knockdown time could be determined. Fourth, if this test would focus more on identifying a strongly resistant population rather than discriminating between susceptible and resistant altogether, a discriminating knockdown time would be more accurate and useful for the grain manager who may have strongly resistant beetles that are difficult to control. It has become clear through dose response assays and in some limited field observations (Collins 1998) that the problem in controlling resistant insects does not lie within the low frequencies of weak resistant beetles in a population, but with the strong resistance phenotypes (Opit et al. 2012). Last, a commercial quick test should warn users not to allow the bioassay to be disturbed during the trials. If test populations are disturbed while being exposed to the phosphine, data could be skewed and lead to false conclusions.

Overall, narcosis/knockdown is an interesting method for potential use in surveying for resistance for the *T. castaneum*. This pest species has shown that it is capable of becoming almost intolerant to normal levels of phosphine in what would be considered good fumigations, necessitating action (Opit et al. 2012). Not only is surveying necessary for control of this pest species using phosphine, but surveying tools that allow rapid action are important for real-time decision making. Using the findings from this study, a commercially viable quick test that discriminates between controllable, and non-controllable populations is possible.

## References

- 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.
- Bell, C.H., N. Savvidou, K. A. Mills, S. Bradberry, and M. L. Barlow. 1994.** A same-day test for detecting resistance to phosphine, pp 41-44. *In* Proceedings of the 6<sup>th</sup> International Working Conference on Stored-product Protection. 17-23 April, Canberra, Australia. CAB International, Wallingford, United Kingdom.
- 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.
- Collins, P.J., 1998.** Resistance to grain protectants and fumigants in insect pests of stored products in Australia. *In* Banks, H.J., Wright, E.J., Damcevski, K.A. (Ed.). Stored Grain in Australia, pp. 55–57. *In* Proceedings of the Australian Post-harvest Technical Conference. Canberra, 26–29 May 1998, CSIRO, Canberra, Australia.
- 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.
- Hagstrum D. W., T. W. Phillips, and G.W. Cuperus. 2012.** Stored Product Protection. Kansas State University. Manhattan, KS. KSRE Publ; S–156; 2012.

- Hori, M. and Y. Kasaishi. 2005.** Development of a new assay method for quickly evaluating phosphine resistance of the cigarette beetle, *Lasioderma serricornis* (Fabricius) (Coleoptera: Anobiidae), based on knockdown of the adult beetles. Appl. Entomol. Zool. 40: 99-104.
- 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.
- 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 6<sup>th</sup> International Working Conference on Stored-product Protection*, 17-23 April 1994, Canberra, Australia. CAB International, Wallingford, United Kingdom.
- 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.
- 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 6<sup>th</sup> International Working Conference on Stored-product Protection*, 17-23 April 1994, Canberra, Australia. CAB International, Wallingford, United Kingdom.
- 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.

**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., 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.



**Table 3.1 Mean knockdown time (KT) in minutes for populations of adult *T. castaneum* assayed as single beetles or as groups of 10 for knockdown of 50% or 100% of the group.**

<b>Population<sup>3</sup></b>	<b>FAO % Resistance</b>	<b>Single KT (± SE)<sup>1,2,3</sup></b>	<b>50% KT (± SE)<sup>1,2,3</sup></b>	<b>100% KT (± SE)<sup>1,2,3</sup></b>
<b>USDA</b>	0	8.46± 0.66 A,a	5.19± 0.21 A,b	9.13± 0.46 A,a
<b>Ex, MO</b>	0	10.82± 1.04 A,a	6.13± 0.48 A,a	17.05± 3.01 AB,b
<b>Calgary</b>	0	9.24± 0.42 A,a	7.27± 0.11 A,a	16.23± 1.21 AB,b
<b>Davis</b>	0	14.13± 0.84 AB,a	9.16± 0.84 AB,a	34.83± 7.42 AB,b
<b>Arbuckle</b>	0	14.37± 1.35 AB,a	10.00± 0.30 B,a	31.28± 3.12 AB,b
<b>Williams</b>	0	10.91± 1.01 A,a	10.05± 0.53 B,a	26.16± 2.15 AB,b
<b>Thai</b>	15	14.62± 2.00 AB,a	7.89± 0.29 AB,a	29.17± 3.92 AB,b
<b>Abilene</b>	39	16.14± 1.21 AB,a	11.50± 0.61 BC,a	37.30± 3.84 AB,b
<b>Russell</b>	41	16.82± 1.38 AB,a	14.38± 0.42 CD,a	62.78± 9.12 BC,b
<b>Minneapolis</b>	89	24.47± 2.35 B,a	16.98± 1.04 D,a	100.20± 11.77 C,b
<b>Mitchell</b>	93	19.32± 1.54 AB,a	14.55± 0.87 CD,a	172.15± 24.72 D,b
<b>Red Level</b>	100	52.70± 7.14 C,a	24.33± 2.05 E,a	194.13± 22.13 D,b

<sup>1</sup>Replications per KT type as follows: Single (10), KT<sub>50</sub> (5), and KT<sub>100</sub> (5).

<sup>2</sup>Means for KT results in a row for the same population followed by the same lower case letter, and those in a column comparing among populations followed by the same upper case letter are not significantly different according to a Tukey HSD post hoc analysis.

<sup>3</sup>All differences determined by an ANOVA: Population – F= 92.952, p<0.001 and KT – F =407.484, p<0.001.

Figure 3.1 Regression of knockdown times for individual beetles with discriminating dose frequencies of phosphine resistance for populations reported in Table 3.1.

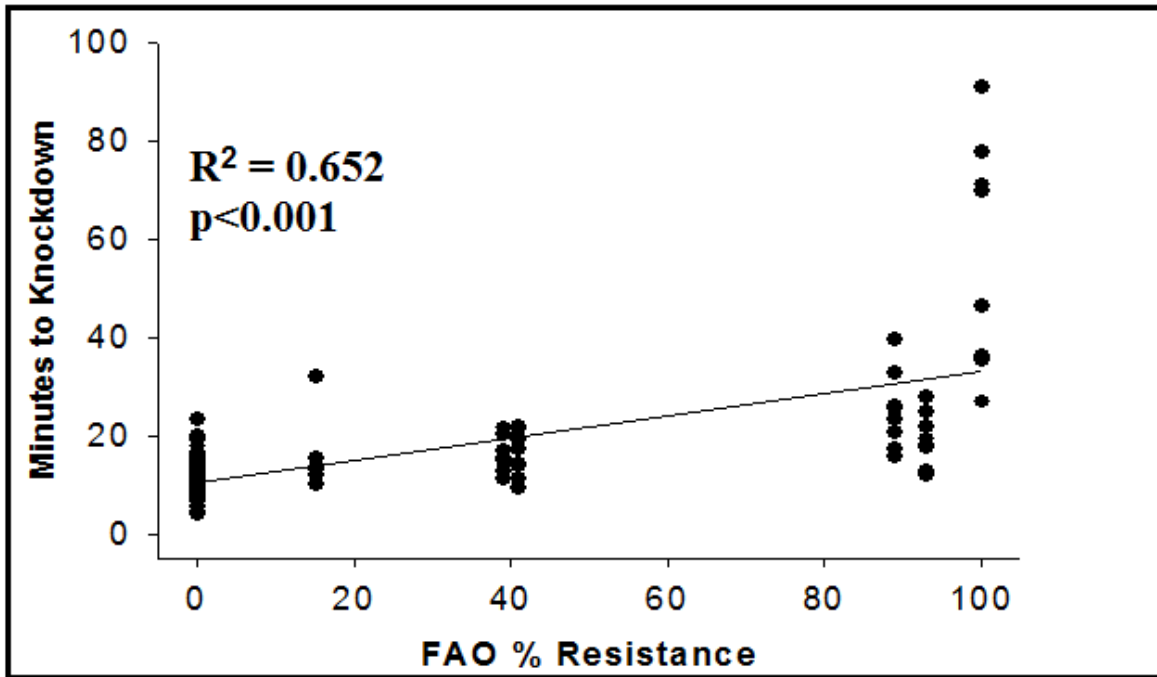


Figure 3.2 Regression of time to knockdown of 50% of beetles in a sample with discriminating dose frequencies of phosphine resistance for populations reported in Table

3.1.

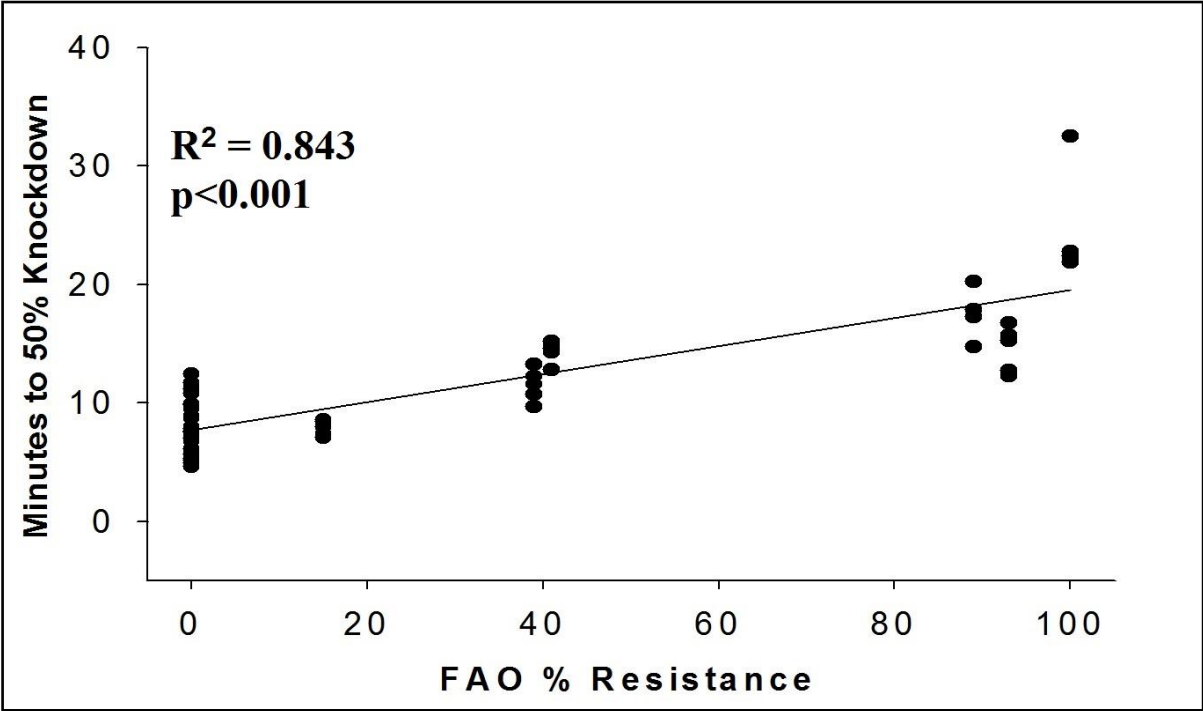
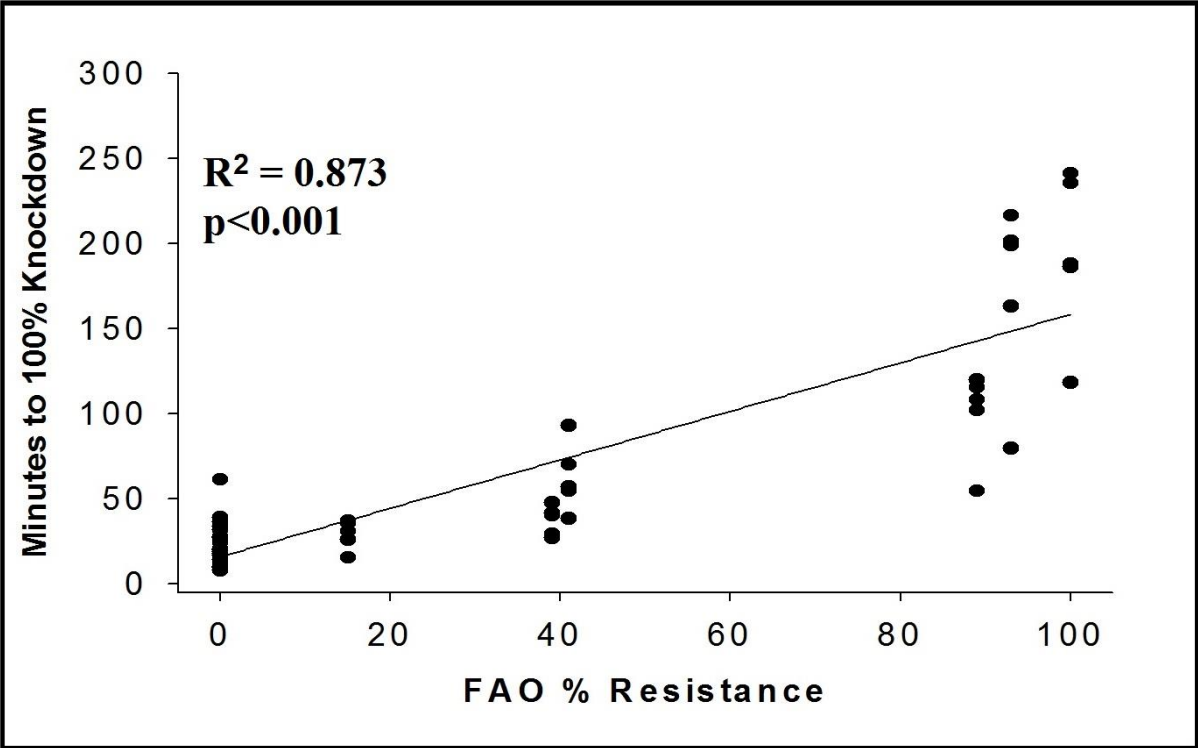


Figure 3.3 Regression of time to knockdown of 100% of beetles in a sample with discriminating dose frequencies of phosphine resistance for populations reported in Table

3.1.



**Figure 3.4 Mean (SE) time to knockdown for individual *Tribolium castaneum* from susceptible and resistant populations exposed to approximately 3000 ppm phosphine when stimulated or not stimulated during observations.**

