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A rapid assay for the detection of resistance to phosphine in the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae)

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ABSTRACT

Resistance to the fumigant phosphine in stored product insect pests is a global problem. Diagnosis of resistance relies on a bioassay developed by the FAO that involves a mortality assessment after 20-h fumigation of a pest population at a discriminating concentration of gas, followed by a 14-day post fumigation assessment. This bioassay is impractical for monitoring and early detection of phosphine resistance in routine pest management. We utilized the procedure of a commercial resistance detection test kit for rapid detection in field populations of lesser grain borer, *Rhyzopertha dominica* (F.). We established a knockdown effect of either susceptible or resistant insects by exposing them to a high concentration of phosphine. We assessed the relationship between adult knockdown times and the FAO method for 18 beetle populations utilizing knockdown criteria for a single beetle in a chamber, or for 50% or 100% knockdown times for groups of beetles, exposed to 3000 ppm of phosphine. We also determined the most effective concentrations that would elicit the quickest knockdown while estimating the recovery times from exposure. Results suggest that a KT_{100} test was better than the KT_{50} and the KT_{single} tests. Based on the responses of susceptible populations, we established that a KT_{100} of approximately 18 min can be used as a viable knockdown time to distinguish a susceptible from a resistant populations. Higher concentrations of phosphine significantly elicited a quicker recovery in strongly resistant populations compared to susceptible populations. These findings have potential for developing a robust commercial kit for practical phosphine resistance detection in populations of *R. dominica* by commercial fumigators, and could be incorporated in a resistance management program.

1. Introduction

Storage of cereal grains and legumes requires that infestation by insects and other pests whose populations have reached a critical action threshold be effectively controlled or prevented as the population increases (Subramanyam and Hagstrum 2012). The lesser grain borer, *Rhyzopertha dominica* (Coleoptera: Bostrichidae), is among the most damaging pests of stored wheat and rice worldwide, with both adults and larvae feeding on germ and endosperm of the target grain and reducing kernels to powder (Gundu Rao and Wilbur 1972). Several methods have been employed to control the lesser grain borer, with fumigation being the most effective method (Edde 2012). The fumigant hydrogen phosphide (PH_3), commonly referred to as phosphine, is a widely used toxin that has been in use for over half a century due to its ideal properties. The solid formulations of phosphine are easy to apply and usually less expensive than other available commercial fumigants. Moreover, its effectiveness against a range of invertebrate and vertebrate pest species and its global acceptance as a residue-free treatment for treated commodities, makes it an important tool in the management of insects associated with stored commodities (Chaudhry 2000). However, these ideal qualities of phosphine have led to an increased dependence by industry and the constant and long-term use of this single fumigant has resulted in selection and establishment of resistance in major pest species (Nayak et al., 2020).

A global survey was undertaken during 1972–1973 by the United Nations' Food and Agriculture Organization (FAO) to assess

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the presence or absence of resistance to phosphine in major pest species by using a discriminating dose bioassay (FAO, 1975). The results of the survey showed that about 10% of the collected populations contained phosphine resistant individuals (Champ and Dyte 1976). After this FAO survey, there have been reports of wide-spread cases of phosphine resistance in several species of stored-product insects in many countries (Collins et al., 2005; Opit et al., 2012; Nayak et al., 2013). In the early 2000s it was reported that phosphine resistance was prevalent in at least 11 species of stored-product insects in 45 countries, with the list growing (Chaudry 2000; Nayak et al., 2020).

Detection of phosphine resistance in stored product insect populations relied on the so-called FAO method (FAO 1975). The FAO method recommends the exposure of adult insects to a discriminatory dose of phosphine for 20 h at 25 °C. After this period the test insects are held in clean air with food for 14 days to allow for recovery or delayed mortality, at which time a final mortality is recorded. Insects that survive this 20-h exposure and the 14-day post fumigation period are considered as resistant while those not surviving are diagnosed as susceptible.

Research in Australia has identified two genetically distinct phosphine resistant phenotypes for *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (Coleoptera: Bostrichidae), referred to as the strong and weak phenotypes (Collins et al., 2005; Schlipalius et al., 2002). Analysis of these insect species determined that mutations at two genetic loci conferred resistance in both *R. dominica* and *T. castaneum* (Jagadeesan et al., 2012; Mau et al. 2012a, 2012b). The first gene, rph1, is responsible for weak resistance, whereas the second, rph2, acts synergistically with rph1 to confer strong resistance (Schlipalius et al., 2002). The "weak" resistance phenotype may require phosphine concentrations of 10-fold−50-fold greater than those needed to kill susceptible beetles for control, while beetles with the "strong" resistance phenotype may require 100-fold or greater concentrations for control relative to susceptible beetles (Nayak et al., 2015; Afful et al., 2020).

The FAO method has been successful in determining the presence or absence of resistance in a given insect population. However, one limitation for using the FAO method to help with pest control decisions is the 14 days needed to ascertain the presence or absence of resistance in a pest population. Additionally, this method is laborious and requires technical expertise and expensive instrumentation for commercial use, such as gas chromatography and methods to generate phosphine in a laboratory for the test. The FAO test, while considered an excellent research tool, requires special training and handling of equipment that are impractical for grain managers or commercial fumigators operating in the grain marketing industry. These limitations of the FAO method have led to research on a simple one-day 'rapid' or 'quick' test for phosphine resistance that is practical and inexpensive.

Several "quick test" studies for phosphine resistance are based on a behavior of adult insect on exposure to high levels of phosphine, known as narcosis, an incapacitation that is assessed by observing and recording treated adult knockdown that usually does not cause death (Bang and Telford 1966; Winks 1985; Reichmuth 1991). When normal susceptible insects are exposed to phosphine at a high concentration (around 0.4 mg/L, approximately 300 ppm, or higher) they enter a narcotized state wherein most of the insects will become inactive and fall over, and then can be revitalized if phosphine is removed from the enclosure and replaced with fresh air (Reichmuth 1991). Based on this behavior, resistant insects are expected to tolerate longer fumigation times before being knocked down, and susceptible insects should be knocked down at shorter exposure times. Research in Australia has used this 'knock down' approach to develop quick tests to diagnose two levels of resistance (weak and strong) in key pest species. Nayak et al. (2013) developed a quick test for *C. ferrugineus*, which allows for a diagnosis of weak and strong resistance within 5 h of exposing the adult insects to 1440 ppm of phosphine. Moreover, for *Sitophilus oryzae*, (Coleoptera: Curculionidae) Nayak et al. (2019) established two quick tests that enable the determination of weak and strong resistance, within 3 h at 1440 ppm and within one and half hours at 3600 ppm.

Several elements are required to develop a robust rapid assay for phosphine resistance, particularly that it is relevant to testing pest populations to help with commercial fumigation decisions. Earlier studies identified the level of phosphine concentrations, the time to knockdown, susceptibility or otherwise of the insect population and what defines a knockdown or narcosis (Reichmuth 1991; Bell et al., 1994; Cao and Wang 2000; Winks, 1984, 1985; Waterford and Winks 1994). Commercially, the only quick test designed for use by grain fumigators is that formulated based on a study by Steuerwald et al. (2006) (Degesch Inc. http://www.degeschamerica.com). That study of the so-called Degesch test kit investigated the time to knockdown within 30 min of adult insects for the most common stored-product pest insects exposed to a high phosphine concentration of 3000 ppm. The test-kit includes an inexpensive plastic 100 ml syringe as an observation knockdown chamber, a 5-L flexible plastic canister to hold newly generated PH_3, a syringe with a rubber hose and two special magnesium phosphide pellets that generated about 4000−6000 ppm of phosphine gas that could then be diluted to 3000 ppm for testing. The Steuerwald et al. (2006) study did not investigate knockdown assessments associated with the lesser grain borer, *R. dominica*. Recently, Athanassiou et al. (2019a) evaluated the mobility patterns of two strains of *T. castaneum* (susceptible vs. resistant to phosphine) at 1000 and 3000 ppm of phosphine after the short interval of 90 min by using the "Detia Degesch Phosphine Tolerance Test Kit." Also, Athanassiou et al. (2019b) estimated the resistance of various populations of 13 stored-product insect species originating from the USA, Greece, Australia and Spain by following a quick diagnostic test that is based on the Detia Degesch Phosphine Tolerance Test Kit.

In a recent study (Afful et al., 2018), we used the FAO assay to determine the levels of phosphine resistance in populations of *R. dominica* collected from 34 locations in North America. Thirty-two of the 34 locations sampled reported varying levels of phosphine resistance in *R. dominica*, suggesting a growing phosphine resistance problem for this species in North-America. We used 18 populations established from that survey (Afful et al., 2018) for the current study described below with the main aim to assess and improve the ability of a commercial phosphine resistance quick test to produce results as reliable as those delivered by a discriminating dose bioassay. The research here addresses the following objectives:

1. Assess the concentrations of phosphine needed to elicit the quickest knock down times using adult *R. dominica* from populations previously classified as susceptible, weakly resistant and strongly resistant.
2. Study the effect of a sustained physical stimulus on knockdown times for adult beetles.
3. Determine the most efficient knockdown time technique for single, or groups (at either 50% or 100% knockdown) of adult *R. dominica* populations in comparison to the mortality data from FAO method.
4. Evaluate the relationship between knockdown time and recovery time at a given phosphine concentration for *R. dominica* populations previously classified as susceptible, weakly and strongly resistant (Afful et al., 2018).

2. Materials and methods

2.1. Rhyzopertha dominica rearing

Twenty *R. dominica* populations were used for this study. Three of these populations, USDA, Wamego and Belle Glade, had been previously categorized as susceptible, weak and strong resistant, respectively, and were selected for extra analysis in the current work. The lab susceptible reference strain, referred to as USDA, originated from a laboratory population maintained at the United States Department of Agriculture (USDA), Center for Grain and Animal Health Research, Manhattan, Kansas, for over 40 years. Stock cultures of other insects used originated from field-collections and had been propagated in the lab for two years following rearing methods as described previously (Afful et al., 2018). Briefly, adult beetles from each population were accumulated in 473-ml wide-mouth glass Mason jars (Hearthmark, LLC, Fishers, IN), closed with a ventilated lid to prevent the adults from escaping the jar, but allowing air and moisture exchange. Beetles were fed with a diet consisting of a mixture of 95% whole wheat kernels and 5% wheat flour and placed in a growth chamber kept at a constant regime of 28 °C, 65% relative humidity (RH) and a photoperiod of 16:8 (L:D) h until adult offspring were used in the quick test assay.

2.2. Phosphine generation and exposure chamber

We used PYREX® 55 ml Screw Cap Culture Tubes with PTFE-lined phenolic caps as a fumigation chamber that was intended to mimic the 100 ml syringe exposure chamber of the Degesch test kit (Steuerwald et al., 2006; pictured in Fig. 2 of Cato et al., 2019). The glass tube measured 25 × 150 mm and a gas-tight Fisherbrand™ Turnover Septum Stopper served as the inlet to introduce phosphine gas. We performed two experiments to assess the effect of phosphine concentrations on knockdown times. In the first experiment we tried to imitate the methods employed by Steuerwald et al. (2006) for which a phosphine concentration of 3000 ppm was used in testing the time to knockdown of *R. dominica* adults. A Hamilton® 25 ml, Model 1025 TLL gas tight Syringe was used to transfer 16.5 ml of 1% (10,000 ppm) phosphine to fumigation chambers from gas tight CEL Scientific Tedlar® PVF film bags contained the 1% phosphine obtained from a cylinder. This volume of 1% phosphine was determined to bring the concentration within fumigation chambers to approximately 3000 ppm. In the second experiment we assessed the use of five different exposure concentrations (500,1000, 2000, 3000 and 5000 ppm) on the knockdown times using adults from the three *R. dominica* populations previously characterized as susceptible, weak and strong resistant phenotypes (Afful et al., 2018). The volumes needed for the fumigation jars for each concentration were 2.75 ml, 5.5 ml, 11 ml, 16.5 ml and 27.5 ml; to achieve the respective five target phosphine concentrations Analysis of the fumigation chambers after the injection of the gas was undertaken by the quantitative GC-FPD method described below.

2.3. Quantitative GC analysis

Phosphine concentration in each fumigation chamber was determined with a Shimadzu GC-17A (Shimadzu, Kyoto, Japan) gas chromatograph, according to methods described by Cato et al. (2017) and Afful et al. (2018). Quantitative gas chromatography (GC) was facilitated by a GS-Q capillary column (30 m long × 0.53 mm i.d., 0.25 μm film thickness, J & W Scientific, Folsom, CA) and a flame photometric detector set in the phosphorous mode. All gas samples from the fumigation chambers were injected onto the GC with a Hamilton® 25 μL, Model 1702 N SYR, Cemented NDL, 22 ga, 2 in, gas tight syringe. The operating conditions of the GC were as follows: injector temperature of 200 °C, detector temperature of 200 °C, column flow rate of 4 ml/min, and oven temperature of 150 °C in split injection mode. Ultra-high purity helium purchased from Linweld (Lincoln, NE) was the carrier gas. A 200-ppm phosphine standard was made by the dilution of 1% phosphine mixed into a carefully measured volume of air in a CEL Scientific Tedlar PVF film bag. Injections of this standard gas at volumes of 25, 20, 15, 10, and 5 μl were analyzed to generate a standard curve for quantitative analyses. We set the 15-μl injection equivalent to the standard of 200 ppm.

2.4. Comparison between FAO and knockdown time method

We studied three different methods to perform a rapid knockdown assay for adult *R. dominica*: the time to knockdown of a single beetle in a tube, the time to knock down 50% of a group of beetles in a tube (KT_{50}) and the time to knock down 100% of the beetles in a tube (KT_{100}). Adults from 18 *R. dominica* populations with previously determined FAO phosphine resistance frequencies (Afful et al., 2017) were assessed using these three knockdown methods. Each method used a phosphine concentration of 3000 ppm as reported by Steuerwald et al. (2006). In testing for KT_{50} and KT_{100}, 10 insects from each population were tested in 5 replications each. The definition of knockdown time is that utilized by Cato et al. (2019), who explained it as the time at which there is a persistent and complete lack of movement by a treated insect. Time to knockdown for each assay of groups was recorded when there was a complete lack of movement in either 50% or 100% of the group of beetles in a fumigation tube for at least 30 s. In the single-beetle experiment we used one insect per replication ($n = 10$). Knockdown times for all the tested single insect trials, the KT_{50} trials and the KT_{100} trials were compared against the known FAO frequencies (from Afful et al., 2018) using linear regression. One-way ANOVA was used to show if there were differences among the three knockdown time techniques tested and among the *R. dominica* populations within a technique ($P < 0.05$). Means were separated by Ryan-Einot-Gabriel-Welsch multiple range test (REGWQ) (SAS Institute, 2012). Graphs were plotted using SigmaPlot, version 12.5.

2.5. Knockdown and recovery time assay

We assessed the relationship between knockdown time and the time to recover for adults from the three *R. dominica* populations characterized as susceptible (USDA), weak (Wamego) and strong resistant to phosphine (Belle Glade). Recovery time was defined as the time that all knocked down beetles needed to be normally moving and upright and using all their legs. Observation of recovery was made with the aid of a dissecting stereo microscope and recorded for each beetle in the group until the last beetle recovered completely after a recovery time of at least 1 min or longer. The time when the last beetle in a test group had moved for 1 min at a given time after the first beetle's recovery, was recorded as the recovery time for that entire replicate of beetles from the given test population. Each population tested had 10 insects tested in five separate replicates. Five phosphine concentrations, 500, 1000, 2000, 3000 and 5000 ppm were first tested for KT_{100} and afterwards were carefully ventilated in a fume hood for 15 s, after which the time to knockdown was monitored. Two-way ANOVA with the Ryan-Einot-Gabriel-Welsch multiple range test (REGWQ) was used to identify significant variation between recovery time and knockdown time.

2.6. Effect of stimulus during knockdown trials

Our preliminary observations suggested that any beetle in a given tube tends to get stimulated by other beetles in that group when assayed together in the same tube, and this then resulted in a longer time to knockdown compared to a single undisturbed *R. dominica* in a tube. Following methods by Cato et al. (2019), this effect was tested for two previously characterized *R. dominica* populations (Afful et al., 2018). A resistant *R. dominica* population called Junction City 1 with a FAO resistance frequency of 57%, and the USDA susceptible population, were used for this experiment. We used single beetles per tube from the two populations, each receiving a set of treatments we termed "stimulus" and "no stimulus". The stimulus treatment involved the phosphine-treated tubes being held on the bench horizontally with a single beetle being rolled a complete 360° in one direction and then 360° back to the starting position at 1-min intervals until a 30-sec knock down was achieved. A non-stimulus treatment involved not moving the tubes at all during the full duration of the knockdown exposure. Each population, susceptible ($n = 40$) and the resistant ($n = 40$), were ran with "stimulus" and "no stimulus". These two populations were then compared independently using an Unpaired T-Test.

3. Results

Table 1 shows results for the three techniques used in recording knockdown times from 18 populations of *R. dominica* that have been assessed in an earlier study determining frequencies of phosphine resistance using the FAO discriminating dose method (Afful et al., 2018). The single beetle technique, KT_{single} had average knockdown times ranging from 6.01 to 59.52 min among the populations. The knockdown test using KT_{50} had average times ranging from 5.91 to 41.31 min while KT_{100} technique had times that ranged from 10.41 to >300 min. Overall, there were significant differences among results obtained from the three tests for each population and among all populations within each of the tests separately ($p < 0.01$). Further post hoc analyses revealed that among all three techniques used, the KT_{100} was statistically different from results recorded for the other two techniques for in 18 populations assayed. Eight of the 18 populations had KT_{single} and KT_{50} not different from each other with the other 10 knockdown times significantly different.

A similar post hoc analysis was used to determine the differences among the populations within each knockdown technique. The single insect trials revealed that most of the populations were significantly different from each other except for Stirling and Coronach, which recorded average knockdown times of 11.04 min. A similar observation was encountered in Princeton, Parlier 2 and Garden City with an average knockdown time of 19.51 min. These six populations had very similar FAO resistance frequencies. The KT_{50} test resulted in the least variability among the populations, a 6.99-fold difference from the KT_{50} of 5.91 min for USDA to 41.31 min for Belle Glade. The strong-resistant Belle Glade population had a KT_{50} that was significantly different from all other populations in its response to phosphine across all three techniques used. There was not a general trend of groupings as most of the populations differed significantly from each other.

The KT_{100} technique of assessing knockdown time showed the best separation of populations and the difference from susceptible to strong resistant populations was the largest at over 28-fold, from 10.41 min for USDA to 300 min for Belle Glade. Populations that had FAO resistance frequencies from 0 to 4% were significantly different from the other populations with KT times between 10.41 and 17.37 min. The knockdown time of the population categorized as strong resistant, Belle Glade, was significantly different from the other 17 with time of over 300 min. The two populations Parlier 1 and Minneapolis with the highest recorded resistance frequencies from the FAO technique, were also significantly different from the others with an average time of 193.61 min. Results also showed that populations that had FAO resistance frequencies between 71 and 93% were not significantly different from each other with knockdown times ranging from 77.19 to 104.50 min. However, Burleson, which fell in this range of frequencies, was significantly different from these five with a knockdown time of 134.06 min.

Fig. 1 shows regression plots for the raw data on knockdown time collected for each of the three techniques as a function of the FAO resistant frequencies previously determined for these 18 *R. dominica* populations from Table 1. The ability of resistance frequency in a population to explain the time-to-knockdown according to the KT_{single} was estimated with an r^2 value of 0.56

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

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

[a] Populations listed and their corresponding FAO % Resistance values are taken directly from Afful et al., (2017).
[b] Means for KT results in a row followed by the same lower-case letter, and those in a column followed by the same upper-case letter, are not significantly different according to a REGWQ post hoc analysis.
[c] All differences determined by an ANOVA: Population – $F_{range} = 25.11–14219.10$, $p \ll 0.01$ and KT- $F_{range} = 61.32–3300.98$, $p \ll 0.01$.

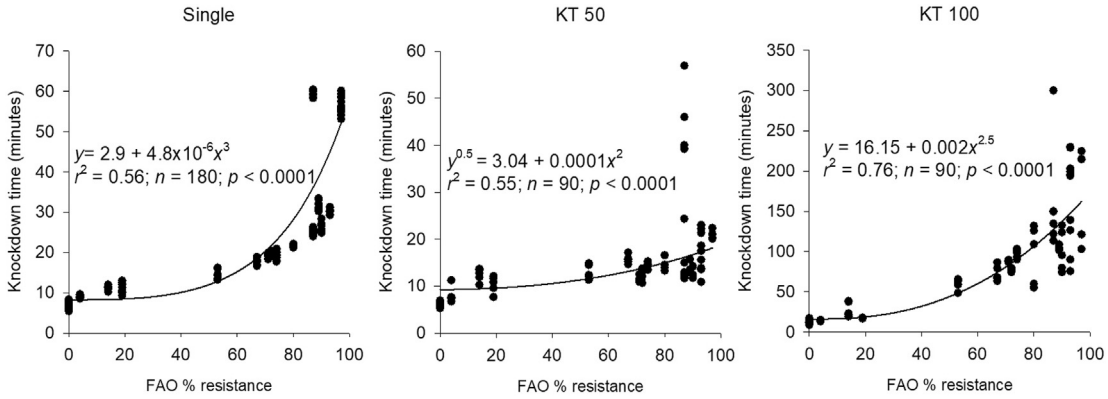

Fig. 1. Regression of knockdown times as a function of pre-determined FAO discriminating dose resistance frequencies for raw data on individual beetles or groups as reported in Table 1.

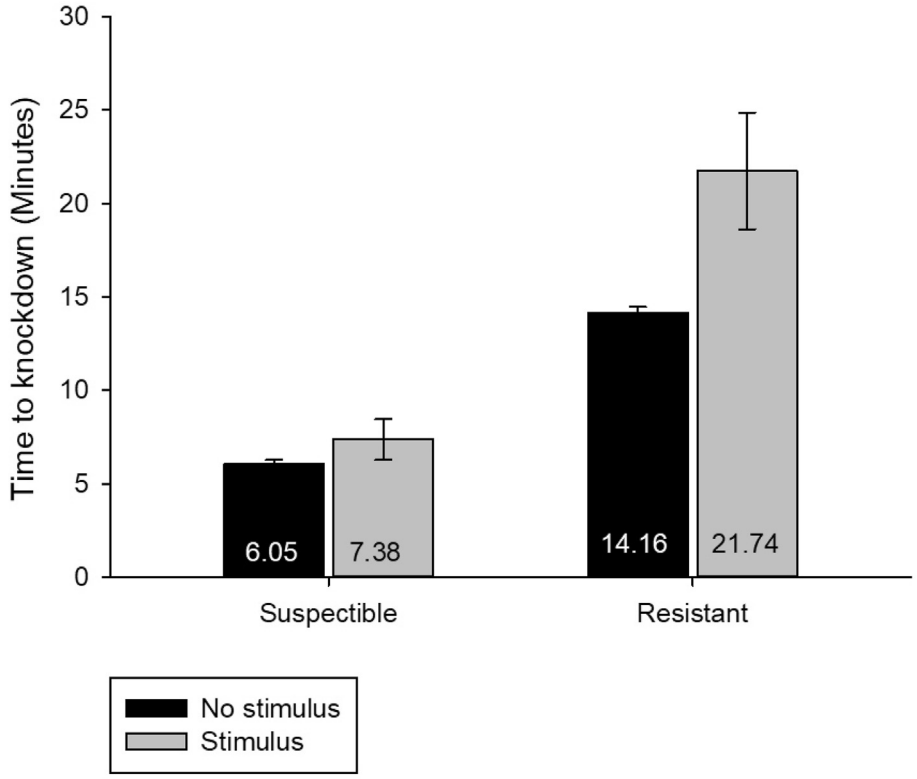

Fig. 2. Mean (SE) time to knockdown for individual *Rhyzopertha dominica* from a susceptible (p«0.01) and a resistant population (p < 0.03) that were either given a physical stimulus or not while being exposed to 3000 ppm phosphine concentration.

($p < 0.0001$) while that of KT_{100} was 0.76 ($p < 0.0001$). The relationship between KT_{50} and FAO resistance frequencies was the least with an r^2 value of 0.55 ($p < 0.0001$).

The role that a physical stimulus played in knockdown time for single beetles from two populations categorized as susceptible and resistant by the FAO assay is shown in Fig. 2. There was a significant difference for both populations between insects treated with stimuli and those untreated (p«0.01 and $p < 0.03$). The mean knockdown time for susceptible beetles without any stimulus applied to the assay vial was 6.05 min (SE = 0.23) (n = 40), while beetles stimulated by rolling the exposure vial took 7.38 min (SE = 0.31) (n = 40) to knockdown. A *t*-test analysis using the raw data showed a statistical difference indicated by a p-value«0.01. The resistant population mean knockdown time recorded a similar trend. While the beetles without stimulus was 14.16 (SE = 1.09) (n = 40) minutes, that with stimulus was 21.74 (SE = 3.12) (n = 40) minutes.

Fig. 3 shows the effects of phosphine concentration on time to knockdown and recovery time for groups of 10 adult *Rhyzopertha dominica* from susceptible, weak and strong resistant populations. Generally, it was observed for all three populations that as the phosphine concentration increased the knockdown times decreased. In the USDA susceptible population the KT started off at 48.85 min at a phosphine concentration of 500 ppm decreased to 26.12 min at 1000 ppm, 21.12 min at 2000 ppm, 10.07 min at 3000 ppm and finally to 7.39 min at 5000 ppm. For beetles from the weak resistant Wamego population the KT started off at 433.20 min at a phosphine concentration of 500 ppm decreased to

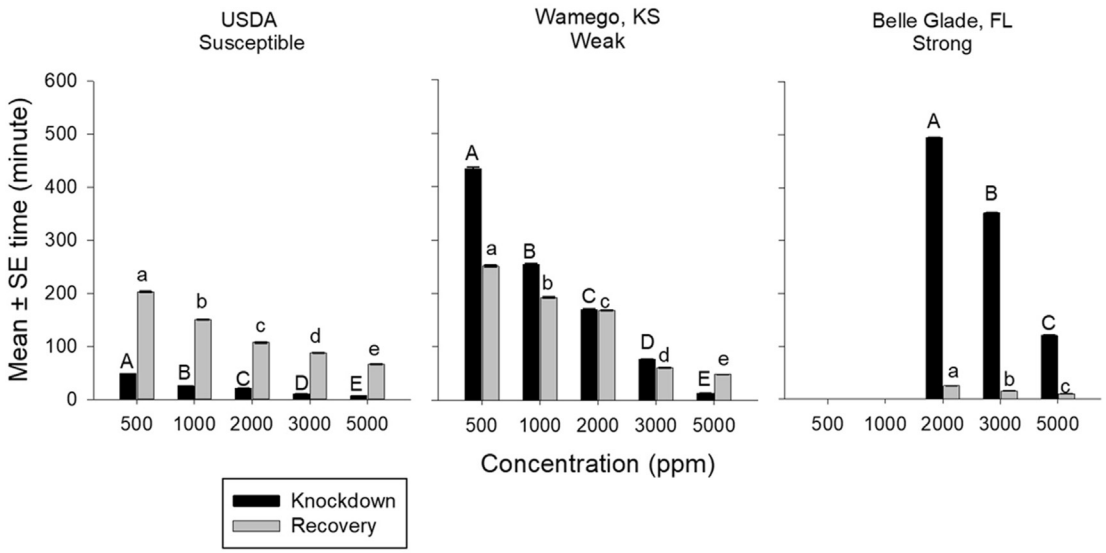

Fig. 3. Mean (SE) time to knockdown and recovery time for group of 10 adult *Rhyzopertha dominica* from susceptible, weak and strong resistant populations exposed to different phosphine concentrations. Statistical analyses revealed KT − F_{range} = 3786.56−200026, p«0.01 and RT- F_{range} = 144.33−10709.5, p«0.01. Mean KTs within a population with the same upper-case letter are not significantly difference ($p < 0.05$) and mean recovery times with the same lower case letter are not significantly different ($p < 0.05$).

254.90 min at 1000 ppm, 170.02 min at 2000 ppm, 77.06 min at 3000 ppm and finally to 13.21 min at 5000 ppm. Lastly the strong resistant population from Belle Glade, FL had no recording of KT at the lower concentrations of 500 ppm and 1000 ppm as insects exposed to these concentrations were actively moving after 72 h, so we terminated that part of the assay. The complete experiments for KT of Belle Glade beetles began at 2000 ppm, which had an average time of 495.5 min, a KT of 352.9 at 3000 ppm and 120.29 min at 5000 ppm. Post hoc analyses revealed each observed knockdown time per concentration was significantly different from each other at P«0.01). The recovery following knock down and ventilation of fumigant are also summarized in Fig. 3. The strong-resistant Belle Glade beetles recovered quickly compared to susceptible and weakly resistant individuals after exposure to the different phosphine concentrations. The recovery time (RT) of the USDA susceptible population started off at 203 min at 500 ppm decreased to 150.70 min at 1000 ppm, 107.50 min at 2000 ppm, 88.02 min at 3000 ppm, and finally, 66.30 min at 5000 ppm. The weakly resistant population RTs included 251.24 min at 500 ppm decreased to 192.54 min at 1000 ppm, 168.23 at 2000 ppm, 61.26 min at 3000 ppm, and finally, 43.80 min at 5000 ppm. The shortest recovery times were recorded in the strong resistant population, which started off at 25.62 min at 2000 ppm, 15.48 min at 3000 ppm and 9.90 min at 5000 ppm. A test to examine the variability between the concentrations showed that there were significant differences among the treatments at p«0.01. Additionally, a post hoc analysis revealed each observed recovered time per concentration was significantly different from each other at P«0.01).

4. Discussion

With the increasing phosphine resistance problem across the globe and the rapid changes in logistics in storages and marketing requirements for insect-free grain, there is a need for a quicker, robust and efficient way of detecting resistance in pests. A simple on-site test for phosphine resistance would provide storage managers an early, accurate and non-technical detection of resistance to make timely decisions on pest management without relying on the lengthy FAO assay. Our study looked at three techniques of assessing knockdown times and a new way of detecting resistance: observing the recovery time. We also compared knockdown times with already established resistance frequencies of 18 geographically distinct populations based on the FAO method to obtain a much clearer understanding on how the techniques could be applied effectively. One factor that this study sought to look at was the effect an external stimulus had on knockdown times. In many rapid assay experiments insects are usually put in groups of 10–20 to assess knockdown times with limited knowledge on the role of an external stimulus on recording knockdown time.

Our results with the KT_{100} assay suggest that taking 1 h to determine if 10 beetles in a group could be completely knocked down is an effective way to detect resistance. The KT_{100} values in Table 1 were all significantly greater than other values from the single beetle KTs and the KT_{50} values, and from a practical standpoint we found it to be an unambiguous and more streamlined approach to wait for the last beetle to be knocked down for a minimum of 30 s. The KT_{100} was able to properly distinguish the most resistant population from the groups categorized as susceptible or with a very low frequency of resistance. Three of the populations tested had FAO resistance frequency of 0%. Steuerwald et al. (2006) established times at which insects needed to be considered knockdown to prove susceptibility. As an example, *Tribolium castaneum* population needed to be knockdown in 8 min or less under a phosphine concentration of 3000 ppm to be considered susceptible. In our study, based on the three identified susceptible populations, we predict that a knockdown time of approximately 18 min or less could be sufficient to categorize a given population of *R. dominica* as susceptible to phosphine, or that any population with a KD_{100} time greater than 18 min could be resistant at some level. Similarly, Athanassiou et al. (2019b) found that laboratory populations of *R. dominica* susceptible to phosphine became 100% knocked down between 4 and 10 min when held at at 3000 ppm.

Results here suggest that longer knockdown times have utility in categorizing resistance phenotypes, particularly strong resistance, with confidence, compared to shorter times, as previously recommended for *C. ferrugineus* (Nayak et al., 2013) and *S. oryzae* (Nayak et al., 2019). The 13 populations in Table 1 with over 50% of

insects scored resistant with the FAO test all had KT_{100} values longer than 60 min. The known strong resistant populations of Minneapolis and Belle Glade had KT_{100} values of 211 and over 300 min, or 3.5 and 5 h, respectively. Categorization of resistance based on KT_{100} is therefore a better technique compared to KT_{50} and K_{single}. Several studies with other species have proposed KT_{50} as an efficient way of scoring phosphine resistance in a population (Cato et al., 2019; Cao and Wang 2000; Waterford and Winks 1994). However, our study supports KT_{100} is the most efficient knockdown test for *R. dominica*, and we recommend that a commercially available quick test like that from Degesch using this KT_{100} metric could be used for *R. dominica*. Other studies have proposed such a metric in the development of rapid assays (Nayak et al. 2013, 2019; Steuerwald 2006; Reichmuth 1991). It is true from our study that the times to knockdown are shorter in both KT_{50} and KT_{single} and can be efficient ways to estimate resistance, but the fact that it is very difficult in distinguishing between the weak and strong phenotypes of resistance from this study makes these unsuitable for use as a metric for a rapid assay.

This study sought to determine if an added physical stimulus during knockdown trials increased the time to knockdown of the beetles. We tried to simulate beetles being moved around, contacting each other, or falling over, so that we could study single beetles having contact stimuli like they would experience in groups with other beetles. Our finding confirms that added stimulus increases the knockdown times of a given population. Although relatively short KTs for single beetles may seem attractive for resistance screening due to the shorter time needed, one drawback is that a single beetle cannot represent a resistance phenotype. Tests with 10 beetles in an exposure chamber may easily allow for observing the range of behavior in a sample due to having 10 beetles rather than one.

Compared to a short range of concentrations used in previous studies (Reichmuth 1991; Waterford and Winks 1994), in our study we looked at five concentrations of phosphine (500, 1000, 2000, 3000 and 5000 ppm) and established that with the increase in concentration there is an acceleration to knock down in both resistant and susceptible *R. dominica*. For strong resistant populations like Belle Glade, using a concentration of about 5000 ppm is more efficient compared to 3000 ppm as the former dose could predict strong resistance at 2 h. Additional findings here are on the utility of recovery time as a new tool to augment knockdown time from a rapid assay to categorize phosphine resistance. We found that as phosphine concentration increased the recovery time was very short for the strong resistant Belle Glade population and longer for the susceptible USDA and weak resistant Wamego populations. Further research with strong resistant *R. dominica* populations may confirm the value of recovery time in identifying strong resistant populations.

Resistance monitoring is an important tool in any IPM program and a rapid assay for the detection of phosphine resistance in *R. dominica* will provide a benefit for the grain industry. A simple test kit like that designed by Degesch could be used to show that samples of *R. dominica* adults with KT_{100} values at less than 20 min could be considered susceptible and suitable for proper treatment with phosphine if the pest population size has reached an action threshold. A population with beetles displaying knockdown times over 30 min but less than 60 min could have the weak resistance phenotype and might be considered for very careful treatment with phosphine at a high concentration for long exposure times (e.g., Afful et al., 2020). However, if samples from a population with high numbers of *R. dominica* needing control have a KT_{100} near or beyond 60 min, then such beetles could likely have the strong resistance phenotype and that bin should best be treated with a pesticide other than phosphine.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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