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From immobilization to recovery: Towards the development of a rapid diagnostic indicator for phosphine resistance

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ABSTRACT

The aim of this work was to evaluate the insect mobility patterns of phosphine-resistant and -susceptible adults of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) after exposure to phosphine. Exposure bioassays were carried out at two concentrations, 1000 and 3000 ppm, while adults were observed every 15 min, for a total period of 90 min. During this observation interval, adults were visually classified as active (able to walk normally), partially immobilized (not able to walk, but showing a minimal movement), or completely immobilized (no visible movement). After the observation period, all adults were placed in a phosphine-free environment, and they again were classified as active, partially immobilized or completely immobilized. At 1000 ppm, the majority of adults of the susceptible *T. castaneum* population were quickly immobilized after a 15 min observation period, while in contrast, the majority of adults of the resistant *T. castaneum* population were still active after the termination of the 90 min interval. At 3000 ppm, the percentage of immobilized susceptible adults was increased at the 15 min observation period, while the majority of resistant adults were immobilized only after 90 min. In the post-exposure period, the vast majority of the susceptible adults were dead. In contrast, most resistant adults recovered, regardless of the concentration that had been exposed. The results of this study delineate major differences in movement in phosphine-resistant and -susceptible *T. castaneum* strains, and can be applied as a quick diagnostic bioassay for the evaluation of resistance to phosphine in stored product insects.

1. Introduction

Phosphine, or hydrogen phosphide (PH_3), is currently the most commonly used gas for insect control in durable stored products (Phillips et al., 2012). Phosphine is generally easier and cheaper to use than most other commercially available gases for commodity treatments, but it causes corrosion of metals, which limits its application in processing facilities (Nayak and Collins, 2008; Phillips et al., 2012). Phosphine has high mammalian toxicity, but this potential hazard can be dealt with using specific safety measures and following best management practices. Although cylinderized formulations of the gas are commercially available, phosphine is usually applied in solid formulations, which are either aluminium phosphide (AlP) or magnesium phosphide (Mg_3P_2) tablets that react rapidly with water to produce phosphine (Phillips et al., 2012). There are numerous studies indicating that, if applied properly, phosphine can effectively control a wide variety of insect and mite species that infest agricultural commodities during post-harvest (Wilkin et al., 1999; Hagstrum et al., 1999; Phillips et al., 2012).

Resistance to phosphine by insects is an increasing problem worldwide and is an issue that cannot be easily mitigated (Phillips et al., 2012). Currently, there are many insect populations that are,

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to different degrees, resistant to phosphine, in many parts of the world (Benhalima et al., 2004; Wang et al., 2006; Nayak et al., 2012; Opit et al., 2012; Kaur et al., 2015; Cato et al., 2017). For example, Benhalima et al. (2004) reported that almost all field populations of the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae), the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) and the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) collected in Morocco included individuals resistant to phosphine. Nayak et al. (2012) showed that several strains of the psocid *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae), the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) and *R. dominica* are resistant to phosphine in Australia. Cato et al. (2017) found different frequency levels of phosphine resistance among 12 out of 25 USA populations of *T. castaneum*. Konemann et al. (2017) reported that all field-collected populations of *C. ferrugineus* in Oklahoma (USA) exhibited resistance to phosphine.

In order to diagnose insect resistance to phosphine, there are several protocols that have been proposed, often with different modifications (Nayak et al., 2012; Kaur and Nayak, 2014; Chen et al., 2015; Collins et al., 2017). Still, accurate diagnosis of potential resistance to phosphine for a given population is an issue that has not been resolved yet, as different protocols will give different results, and the most accurate protocols are labor and time intensive. So far, the most commonly used method for the evaluation of resistance to phosphine is the Food and Agriculture Organization (FAO) test, which, in its "classic" setup, is based on the exposure of the insects that are to be evaluated in jars containing 30 ppm of the gas for 20 h (h) (FAO, 1975; Opit et al., 2012; Cato et al., 2017). However, many research groups have used different modified FAO protocols (White and Lambkin, 1990; Sağlam et al., 2015; Holloway et al., 2016; Cato et al., 2017; Nayak et al., 2017). For instance, Cato et al. (2017) and Nayak et al. (2017) used different phosphine concentrations but kept the 20 h exposure interval for the evaluation of resistance of different populations of *T. castaneum* from North America and Australia, respectively. These modifications to the FAO test render results not directly comparable, which has implications for designing a long-term area-wide strategy for mitigation of resistance to phosphine. Moreover, the FAO test remains a laborious procedure, as it requires insect collection, transfer to a specialized laboratory and placement in airtight jars, injection of the gas, measurement of the gas concentration with gas chromatography (GC), evaluation of survival after 20 h, and then re-evaluation at a certain post-exposure interval, which is usually 7 or 14 days (d) (Sağlam et al., 2015; Opit et al., 2012; Cato et al., 2017). At the same time, there is also a risk of not being able to use the data if there are large deviations from the target concentrations (based on GC measurements) and the possible need to rear the insects over multiple generations, if the initially collected insect numbers are not enough to conduct the bioassays. As a result, currently, the FAO test is mostly used by scientific research groups in surveillance studies of insect resistance, and not by the industry.

One of the quick diagnostic tests that are currently in use is the Detia Degesch Phosphine Tolerance Test Kit (DDPTTK), which has been developed by Detia Degesch GmbH (Laudenbach, Germany). DDPTTK provides a rapid evaluation tool for phosphine resistance, where insects are exposed in syringes that contain a high concentration of gas (e.g. 3000 ppm), and this gas is produced on site by adding tablets into a canister (Steuerwald et al., 2006; Aulicky et al., 2015). In this test, which usually lasts less than 30 min, insect immobilization, not insect mortality, is the indicator of tolerance/resistance (Steuerwald et al., 2006). Apart from being quick, this test can be easily operated by industry personnel, without the need of high specialization equipment.

Tribolium castaneum is a cosmopolitan pest of stored products, particularly abundant in flour and other related amylaceous products (Aitken, 1975), and it is the first agriculturally important pest insect species and coleopteran to have its entire genome sequenced (Tribolium Genome Sequencing Consortium, 2008). Numerous studies document that this species is now resistant to several insecticides with different modes of action, including phosphine (Collins, 1990; Pimentel et al., 2007; Tribolium Genome Sequencing Consortium, 2008; Daglish and Nayak, 2012; Opit et al., 2012; Bajracharya et al., 2013; Cato et al., 2017; Julio et al., 2017). *Tribolium castaneum* has served as a model species in designing diagnostics to evaluate resistance to insecticides (Zhu et al., 2013). For example, it was been recorded that one highly-resistant to phosphine *T. castaneum* strain from Brazil had a transcriptome analysis where the expression of mitochondrial genes is extremely different than that of a susceptible strain (Oppert et al., 2015). In this context, we used this strain, along with a strain that was susceptible to phosphine, to evaluate mobility patterns during and after exposure to DDPTTK, as an effort to correlate insect movement with resistance and improve DDPTTK for use as a field diagnostic product.

2. Materials and methods

2.1. Insects

As noted above, two *T. castaneum* strains were used, one susceptible and one resistant to phosphine. The phosphine susceptible strain is our standard laboratory strain with an LC_{50} (median lethal concentration) of 1.35 ppm; the phosphine-resistant strain originated in Brazil and was used in a previous study (Oppert et al., 2015). The Brazil strain has an LC_{50} and LC_{99} of 309 and 775 ppm, respectively (Oppert et al., 2015). Both species were reared in the laboratory at 27.5 °C and 65% relative humidity (r.h.) on whole wheat flour with 5% brewer's yeast. Adults <3 wk old were used in the tests in this study.

2.2. Bioassays

We used a modified version of the DDPTTK in comparison with the standard procedure that was described by Steuerwald et al. (2006). The kit consists of a 5 l plastic canister, and a 100 ml syringe, and phosphine tablets to generate the gas within the canister. In the instructions for the standard procedure, phosphine gas is generated by the addition of 2 kit tablets to 50 ml of water inside the canister. Adults of a given species to be tested are placed within the syringe and a known concentration of phosphine produced in the canister (usually 3000 ppm, based on the kit instructions) is introduced. Exposed insects are examined for their ability to walk after 8–15 min, depending on the species. If insects are able to move normally after these intervals, they are classified as non-susceptible or tolerant to phosphine.

Based on our modification, twenty adults of each of the tested strain were placed in the plastic syringes, and phosphine was generated as above. We ran two separate sets of bioassays, at two concentrations, at either 1000 or 3000 ppm of phosphine within the syringe. The insects inside the syringe were monitored at 15 min intervals, for a total period of 90 min, and were classified as active (able to walk normally), partially immobilized (not able to walk, but showing minimal movement of tarsi, antennae etc.), or completely immobilized (no visible movement). After the termination of the 90 min observation period, all insects were removed from the syringe and placed in plastic petri dishes with a small quantity of wheat flour. The same procedure was followed by using "control" syringes, i.e. syringes with insects that contained only air. All dishes were placed into an incubator set at 27.5 °C and 65% r.h. and the insects were classified as being active, partially

immobilized or completely immobilized after 2 h, and 1, 2, 3 and 7 d. For each treatment, there were three biological replicates with two technical replicates each.

2.3. Data analysis

To compare the results between the two *T. castaneum* strains, the data for adults that were active, partially immobilized or completely immobilized were analyzed by using the two-tailed *t*-test, at *n*-2 df and 5% level, for each exposure interval and concentration of phosphine. The same procedure was followed for the post-exposure intervals. All analyses were conducted by using JMP 11 (SAS Institute Inc, 2013).

3. Results

3.1. Phosphine exposure period

At 1000 ppm exposure to phosphine, approximately 80% of the phosphine-susceptible *T. castaneum* adults were partially immobilized after 15 min, with only 5% of adults being active (Table 1). Fifteen minutes later, all susceptible adults were either partially immobilized or completely immobilized, with the majority being completely immobilized. All susceptible adults were completely immobilized after 60 min of exposure and remained in this condition until the end of the exposure period. However, less than 10% of phosphine-resistant adults were partially immobilized after 15 min exposure to 1000 pm phosphine. The majority of resistant adults remained active until the end of the exposure period. The number of resistant adults that were completely immobilized was low (average of less than 1 out of 20 beetles exposed) even after 90 min of exposure.

At 3000 ppm, 95% of the phosphine-exposed susceptible *T. castaneum* adults were immobilized (partially or completely) after 15 min of exposure (Table 2). At 30 min, approximately 80% of the susceptible adults were completely immobilized, and 100% were completely immobilized by the end of the exposure period. Interestingly, at 60 and 75 min of exposure, some adults were still classified as partially immobilized, while at 1000 ppm all adults were completely immobilized by this exposure time. For the resistant strain, active adults were notably reduced at 3000 ppm phosphine exposure (Table 2) compared to those at 1000 ppm. After 15 min, approximately 40% of the exposed resistant adults were still active, and activity gradually decreased with increasing exposure time. However, even after 90 min of exposure, an average of three out of 20 adults were still active. The number of resistant adults that were partially immobilized at 3000 ppm gradually increased beyond 15 min, eventually reaching more than 50% due to a gradual decrease of the number of adults that were active, given that the number of adults that were completely immobilized remained stable after 30 min of exposure.

3.2. Post-exposure period

After exposure to 1000 ppm phosphine for 90 min, susceptible *T. castaneum* adults showed little signs of recovery, with few or no active adults observed over the 7 d post-exposure period (Table 3). Some susceptible adults were active at 1 and 2 d post exposure (4% maximum), but these individuals eventually returned to partial or complete immobilization. The number of susceptible adults that were completely immobilized increased post-exposure, reaching 95% after 7 d. At the same phosphine concentration, almost all of the resistant adults were active even at the 2 h post-exposure period, and no adults were partially or completely immobilized at the end of the post-exposure observation period.

After exposure to 3000 ppm of phosphine for 90 min, none of the susceptible adults were active, and most adults transitioned to the complete immobilization state over the post-exposure period, exceeding 95% after 7 d (Table 4). Interestingly, at 1 d post-exposure there was some recovery from complete to partial immobilization, but these susceptible adults did not recover further, and the percentage of partial immobilization gradually declined over the remaining days post-exposure. In contrast, for the resistant strain, almost all of the exposed adults were classified as active even after 2 h post-exposure, and these adults remained active until the end of the observation period, with only 1.5% completely immobilized after 7 d (Table 4).

4. Discussion

Several studies have documented that paralysis of insects, known broadly as knockdown, can be used to characterize susceptibly to a given insecticide. In a recent study, Agrafioti et al. (2015) used a “lethality index” to indicate knockdown of stored product insects to different insecticides, and to examine whether insects that are knocked down are closer to mortality or recovery. In that study, however, the authors used active ingredients with different modes of action, including non-neurotoxic ones, which do not cause knockdown to the exposed insects before death.

The term “narcosis” has been questioned as an accurate term in the case of phosphine, and there were studies where the measurable parameter was referred to as “knockdown” or “inability to

Table 1
Immediate effects of exposure to 1000 ppm of phosphine on two *T. castaneum* strains (phosphine-resistant or -susceptible) measured as the number of adults out of 20 ± SE that were active, partially immobilized, or completely immobilized at 15 min intervals after initial exposure. Within the same effect and exposure, asterisks indicate significant differences between the two strains. In all cases df = 10; two-tailed *t*-test at $P = 0.05$.

Effect	Strain	Exposure (min)					
		15	30	45	60	75	90
Active	Susceptible	1.0 ± 1.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Resistant	18.5 ± 0.4*	18.0 ± 0.3*	17.0 ± 0.7*	14.7 ± 0.6*	14.3 ± 0.7*	13.3 ± 0.8*
	t	−16.1	−69.7	−24.9	−23.9	−21.5	−17.5
	P	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Partially immobilized	Susceptible	17.5 ± 1.2*	7.2 ± 1.2*	1.5 ± 0.7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Resistant	1.5 ± 0.4	1.8 ± 0.3	2.7 ± 0.8	5.2 ± 0.7*	5.5 ± 0.8*	6.0 ± 0.9*
	t	12.5	4.40	−1.10	−7.40	−7.20	−6.70
	P	<0.01	<0.01	0.30	<0.01	<0.01	<0.01
Completely immobilized	Susceptible	1.5 ± 1.0	12.8 ± 1.2*	18.5 ± 0.7*	20.0 ± 0.0*	20.0 ± 0.0*	20.0 ± 0.0*
	Resistant	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2	0.2 ± 0.2
	t	1.50	10.8	34.9	119	119	119
	P	0.17	<0.01	<0.01	<0.01	<0.01	<0.01

Table 2
Immediate effects of exposure to 3000 ppm of phosphine on two *T. castaneum* strains (phosphine-resistant or -susceptible) measured as the number of adults out of 20 ± SE that were active, partially immobilized, or completely immobilized at 15 min intervals after initial exposure. Within the same effect and exposure, asterisks indicate significant differences between the two strains. In all cases df = 10; two-tailed *t*-test at $P = 0.05$.

Effect	Strain	Exposure (min)					
		15	30	45	60	75	90
Active	Susceptible	1.0 ± 1.0	0.0 ± 0.0	0.00 ± 0.0	0.00 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	Resistant	7.5 ± 3.1	5.7 ± 2.1*	4.5 ± 1.7*	4.2 ± 1.6*	3.8 ± 1.5*	3.3 ± 1.4*
	t	−2.00	−2.70	−2.70	−2.60	−2.60	−2.40
	P	0.08	0.02	0.02	0.01	0.01	0.04
Partially immobilized	Susceptible	9.2 ± 2.0	2.5 ± 1.2	1.2 ± 1.0	0.7 ± 0.7	0.0 ± 0.2	0.0 ± 0.0
	Resistant	8.7 ± 2.4	9.2 ± 2.0*	10.3 ± 1.9*	10.7 ± 1.9*	11.0 ± 2.0*	11.5 ± 2.2*
	t	0.20	−2.90	−4.40	−4.80	−5.30	−5.20
	P	0.88	0.02	<0.01	<0.01	<0.01	<0.01
Completely immobilized	Susceptible	10.8 ± 2.0	17.5 ± 1.2*	18.8 ± 1.0*	19.3 ± 0.7*	19.8 ± 0.2*	20.0 ± 0.0*
	Resistant	3.8 ± 2.6	5.2 ± 3.1	5.2 ± 3.1	5.2 ± 3.1	5.2 ± 3.1	5.2 ± 3.1
	t	2.10	3.70	4.20	4.50	4.70	4.80
	P	0.06	<0.01	<0.01	<0.01	<0.01	<0.01

Table 3
Delayed effects of phosphine on two *T. castaneum* strains (phosphine-resistant or -susceptible) after exposure to 1000 ppm of phosphine, measured as the number of adults out of 20 ± SE that were active, partially immobilized, or completely at different post-exposure intervals. Within the same effect and exposure, asterisks indicate significant differences between the two strains. In all cases df = 10; two-tailed *t*-test at $P = 0.05$.

Effect	Strain	Post-exposure				
		2 h	1 d	2 d	3 d	7 d
Active	Susceptible	0.0 ± 0.0	0.8 ± 0.4	0.3 ± 0.2	0.0 ± 0.0	0.0 ± 0.0
	Resistant	19.8 ± 0.2*	18.7 ± 0.5*	20.0 ± 0.0*	20.0 ± 0.0*	20.0 ± 0.0*
	t	−119	−28.0	−93.3	–	–
	P	<0.01	<0.01	<0.01	–	–
Partially immobilized	Susceptible	12.5 ± 2.5*	16.0 ± 2.6*	9.2 ± 1.5*	4.8 ± 1.5*	1.0 ± 0.3*
	Resistant	0.2 ± 0.2	0.8 ± 0.4	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	t	4.90	5.70	6.30	3.30	3.90
	P	<0.01	<0.01	<0.01	<0.01	<0.01
Completely immobilized	Susceptible	7.5 ± 2.5*	3.2 ± 2.6	10.5 ± 1.4*	15.2 ± 1.5*	19.0 ± 0.3*
	Resistant	0.0 ± 0.0	0.5 ± 0.5	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	t	3.00	1.00	7.30	10.5	73.6
	P	0.01	0.34	<0.01	<0.01	<0.01

Table 4
Delayed effects of phosphine on two *T. castaneum* strains (phosphine-resistant or -susceptible) after exposure to 3000 ppm of phosphine, measured as the number of adults out of 20 ± SE that were active, partially immobilized, or completely immobilized at different post-exposure intervals. Within the same effect and exposure, asterisks indicate significant differences. In all cases df = 10; two-tailed *t*-test at $P = 0.05$.

Effect	Strain	Post-exposure				
		2 h	1 d	2 d	3 d	7 d
Active	Susceptible	0.0 ± 0.0	0.0 ± 0.0	0.8 ± 0.5	0.2 ± 0.2	0.0 ± 0.0
	Resistant	18.7 ± 0.4*	19.8 ± 0.2*	19.8 ± 0.2*	19.8 ± 0.2*	19.7 ± 0.2*
	t	−44.3	−119.0	−37.6	−83.4	−93.3
	P	<0.01	<0.01	<0.01	<0.01	<0.01
Partially immobilized	Susceptible	7.3 ± 1.9*	19.0 ± 0.8*	13.5 ± 1.3*	3.7 ± 1.7*	0.3 ± 0.3
	Resistant	1.3 ± 0.4	0.2 ± 0.2	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
	t	3.00	22.6	10.3	2.60	1.00
	P	0.01	<0.01	<0.01	0.03	0.34
Completely immobilized	Susceptible	12.7 ± 1.9*	1.00 ± 0.8	5.70 ± 1.6*	16.2 ± 1.4*	19.7 ± 0.3*
	Resistant	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.2
	t	6.60	1.20	3.40	11.5	49.0
	P	<0.01	0.25	<0.01	<0.05	<0.01

walk normally" (Winks, 1984, 1985; Winks and Waterford, 1986; Woodman et al., 2008; Bo et al., 2010; Aulicky et al., 2015). Winks and Waterford (1986) measured the time to narcosis due to exposure to phosphine in a *T. castaneum* strain and found that phosphine-induced narcosis was not related to resistance. In our study we found different patterns of deviations from normal movement, in phosphine-susceptible or -resistant *T. castaneum* adults, suggesting that differences in transitions from active to minimal movement and from minimal movement to complete immobilization may be an indicator of resistance to phosphine. In general, deviations from "active" were expressed more rapidly, and to a higher percentage, in phosphine-exposed adults from the susceptible strain, and, most importantly, the percentage of immobilized adults increased after only 15 min of exposure. Therefore, in our study, we refer to "partial immobilization" for individuals that had minimal movement, which, in other studies, could be considered as "under narcosis".

Using the data generated in this study, we were interested in

determining a) if the percentage of individuals with not normal movement during phosphine exposure is related to resistance to phosphine and b) if the speed at which this deviation from "active" is induced is related to recovery post-exposure. Winks and Waterford (1986) reported that for a phosphine-resistant strain of *T. castaneum*, the level of resistance was lower at lower phosphine concentrations. Moreover, paradoxically, at higher phosphine concentrations, lethal time was higher (Winks, 1984, 1985), a very interesting phenomenon, which is currently known as the "sweet spot", and is related to insect partial immobilization. The fact that the time-to-mortality for *T. castaneum* increased with increased phosphine concentration (Winks, 1982, 1984) may be related to the increased partial immobilization, which remains at relatively long intervals, and does not change until complete immobilization.

When comparing our phosphine-susceptible strain responses to the low and high phosphine concentration, the end effect was the same after both 2 h and 7 d post-exposure. However, the initial response at 15 min was different, with a higher percentage of completely immobilized adults within 15 min at the higher concentration, and perhaps a slightly slower transition from partial to complete immobilization at 3000 compared to 1000 ppm. Interestingly, the resistant strain responded differently, and appears to have more of a steady level of immobilization, either partial or complete, across time during the 1000 ppm exposure, but a slightly higher number of individuals in each category when exposed to 3000 ppm. We observed a difference between the two doses in recovery of resistant adults after treatment. At 1000 ppm, almost all adults regained complete recovery to active. However, at the 3000 ppm dose, some completely immobile adults transitioned back to active, and some of the partially immobile reverted back to active. Interestingly, after 1 d post-exposure, resistant adults demonstrated good recovery from exposure, but by 7 d many reverted back to partial immobilization, possibly a limitation of their ability to recover fully after exposure to phosphine.

Increasing phosphine concentration increased the percentages of adults that were immobilized for both strains. However, the number of resistant adults that were partially immobilized increased gradually over time, and the number of completely immobilized adults was practically unaffected for most of the exposure period. Therefore, there was a percentage of completely immobilized adults (approximately 25% of the total) which remained stable at this condition during the entire exposure interval (90 min). These differences in these immobilization states indicate observational-based studies of responses to phosphine over short periods of time might be used instead of the longer exposure times currently recommended. For example, with the FAO test, the insects have to remain in the same vial or container for 20 h, while in the current test the bioassay can be terminated at 90 min, and even shorter exposure periods may also be informative. The simplicity and the short duration of the exposure period in the current test makes it easier to be used before a fumigation, so that exposure times and concentrations may be adjusted to deal with resistance levels in the local population (Aulicky et al., 2015).

Another advantage of the approach in the current study is that shorter exposure times enable follow-up studies to be conducted on the same insects that were exposed, which is useful for selection and genetic analysis. Further research is needed using shorter total exposure periods to confirm that these individuals will recover, but if this trend holds, these short exposures can be used to select individuals for physiological and functional genomics research on resistance and for selection experiments. In the same way, measurements could be used to also detect the frequency of resistance mutations in groups of adults that may be missed by simply evaluating mortality after longer exposure to phosphine. Thus, apart from being easy-to-use on site, the DDPTTK can be also used for research purposes.

Despite the fact that the current label instructions for the DDPTTK do not recommend any post-exposure observation, the results of the present work suggest that this observation is also important. For the susceptible strain, it became evident that partial immobilization was high even after 15 min of exposure, and, most importantly, it was gradually replaced by complete immobilization. In fact, for this strain, the adults that were found immobilized up to the end of the observation period (7 d) were dead, which means that immobilization at the end of the exposure period is mortality. This phenomenon was observed despite the short post-exposure recovery period and indicates that the effects of short exposures to phosphine in individuals from the susceptible strain were irreversible. Moreover, even if there were some individuals of the susceptible strain that were still alive after the exposure interval, delayed mortality rapidly occurred after their removal from the syringe. The converse was true for the resistant strain, as in this case immobilization was followed mostly by recovery rather than by mortality. The recovery appeared to be related to the speed at which partial immobilization occurred, and thus the short term observations of immobilization could predict both responses. Consequently, we consider that recovery during the post-exposure period is a reliable indicator of resistance, at least at the exposure intervals and the concentrations for the insect strains observed in this study, especially when combined with immobilization patterns during the exposure. When insects become immobilized at low phosphine concentrations, they do not respond even when they are disturbed, but there are certain immobilization thresholds on which disturbance causes a short activity interval (Winks, 1985). Immobilization, at least in the case of resistant insects, does not necessarily mean death, but is more likely to lead to death for susceptible individuals. Hence, a slower speed to partial immobilization, not complete immobilization, is an indicator of resistance. Moreover, the post-exposure behavior of the immobilized adults is equally important. Using the data of the strains tested here, quick immobilization means slow recovery or no recovery, and conversely, delayed immobilization leads to quick recovery.

In our bioassays, we used a fixed 90 min exposure to evaluate phosphine resistance, and observed that the vast majority of resistant adults recovered, regardless of the concentration and the post-exposure period. For the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae), Nayak et al. (2012) developed a rapid bioassay test of approximately 5 h that gives a clear indication of the resistance status of the tested populations. At the post-exposure period of 3000 ppm for the susceptible strain, there was an initial increased percentage of insects that had been immobilized, considered as a direct consequence of the increased numbers of immobilized adults at the end of the 90 min exposure period.

In our study, we observed that differentiations between phosphine-susceptible and -resistant adults, i.e. deviations from normal movement of adults, were evident even after 15 min of exposure at 3000 ppm, which can be further considered as the "critical threshold" for resistance of *T. castaneum* populations for the DDPTTK. In contrast, these deviations were less defined at 1000 ppm, and time-to-immobilization was longer than that for 3000 ppm. We also found that time-to-immobilization was inversely proportional to time-to-recovery of the same individuals, and this characteristic also can be considered as an indicator of resistance. It should be noted however, that our observations may differ in cases of other species that are not particularly mobile like *T. castaneum*, for example, *R. dominica*. Further experimentation is required with a broader spectrum of species and strains to illustrate potential "critical exposure" periods and "speed to immobilization"

levels that may be utilized as quick diagnostic tools for resistance to phosphine. In our work, we examined two "extremes", i.e., one highly susceptible and one highly resistant strain, so it would be useful in the future to provide data on strains with "intermediate resistance frequency".

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