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Using immobilization as a quick diagnostic indicator for resistance to phosphine

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ABSTRACT

In the present study, we evaluated a quick diagnostic test for resistance to phosphine in stored-product beetle species. We collected different populations of thirteen species, obtained from different laboratories in different counties, i.e., USA, Greece, Australia, Germany and Spain. There were also tested populations that have been sampled from different facilities (field populations). We used the Detia Degesch Phosphine Tolerance Test Kit (DDPTTK), which is based on the exposure of the insects on a high concentration of phosphine for shorter exposure periods. The tested concentrations to phosphine were 1000 and 3000 ppm. Briefly, 20 adults of the tested populations were placed in a 100 ml plastic syringe. The observations were taken every 2 min and the exposed adults were classified as either walking normally or being immobilized (knocked down), i.e., not walking normally. In light of our findings, the time to reach knockdown of all adults was notably reduced at 3000 ppm in comparison with 1000 ppm. For the majority of the species and laboratory populations tested, at 3000 ppm, the time required for knockdown ranged between 8 and 14 min. In contrast, for some of the field populations, knockdown did not reach 100% even after 300 min of exposure, at either 1000 or 3000 ppm. Based on the results of the present study, we recommend that the DDPTTK can be operated at 3000 ppm, and we provide the critical threshold times per species for the characterization of tolerance/resistance.

1. Introduction

Resistance to phosphine has been recorded and quantified from many parts of the world, and is considered an issue of major importance for the continued effectiveness of this gas in stored product insect pest management (Collins et al., 2005; Nayak et al., 2015; Afful et al., 2017). For many major stored-product insect species, “weak resistance” has been reported, and seems to be the most common type of resistance to phosphine (Nayak et al., 2015; Afful et al., 2017). Recently, so called “strong resistance” has developed and has appeared in Australia for the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) (Collins et al., 2005), the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae) (Holloway et al., 2016), and the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Leamophloeidae) (Nayak et al., 2012, 2013). Similar findings of strong resistance have been reported from other parts of the world, such as China (Song et al., 2011), India (Rajendran and Narasimhan, 1994; Kaur et al., 2015), Morocco (Benhalima et al., 2004), Brazil (Pimentel et al., 2009; Lorini et al., 2007; Pimentel and Guedes, 2010), USA (Opit et al., 2012; Chen et al., 2015; Sağlam et al., 2015; Afful et al., 2017) and Pakistan (Alam et al., 1999; Ahmad et al., 2013).

There is a range of methods available for the evaluation of phosphine resistance. Schlipalius et al. (2012) identified the mutations that are responsible for both types of resistance, which can be used with success for the molecular diagnosis of resistance in populations. Chen et al. (2015) also presented a molecular diagnostic protocol based on specific markers for the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *R. dominica*. Nevertheless, these diagnostics are generally laborious

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Table 1
Different populations of stored product beetle species, obtained from different laboratories and areas throughout the world.

Species	Strain Code	Origin	Rearing media
T. castaneum	*USDA*	CGAHR (Laboratory)	Wheat flour plus 5% brewer's yeast
T. castaneum	*UTH*	LEAZ (Laboratory)	Wheat flour plus 5% brewer's yeast
T. castaneum	*TC4*	QDAF (Laboratory)	Wheat flour plus 5% brewer's yeast
T. castaneum	*JKI*	FRCCP (Laboratory)	Wheat flour plus 5% brewer's yeast
T. castaneum	*BTS*	Serbia	Wheat flour plus 5% brewer's yeast
T. castaneum	*INJ*	Serbia	Wheat flour plus 5% brewer's yeast
T. castaneum	*3DUB*	United Arab Emirates	Wheat flour plus 5% brewer's yeast
T. confusum	*USDA*	CGAHR (Laboratory)	Wheat flour plus 5% brewer's yeast
T. confusum	*UTH*	LEAZ (Laboratory)	Wheat flour plus 5% brewer's yeast
T. confusum	*D1AGR*	Cyprus	Wheat flour plus 5% brewer's yeast
S. oryzae	*USDA*	CGAHR (Laboratory)	Wheat
S. oryzae	*UTH*	LEAZ (Laboratory)	Wheat
S. oryzae	*IRTA*	IRTA (Laboratory)	Wheat
S. oryzae	*3TAB*	Germany	Wheat
S. oryzae	*BPSIL*	Serbia	Wheat
S. oryzae	*ASC11*	Greece	Wheat
S. granarius	*USDA*	CGAHR (Laboratory)	Wheat
S. granarius	*UTH*	LEAZ (Laboratory)	Wheat
S. granarius	*IRTA*	IRTA (Laboratory)	Wheat
S. zeamais	*USDA*	CGAHR (Laboratory)	Maize
S. zeamais	*IRTA*	IRTA (Laboratory)	Maize
S. zeamais	*MACH*	Brazil	Maize
R. dominica	*USDA*	CGAHR (Laboratory)	Wheat
R. dominica	*UTH*	LEAZ (Laboratory)	Wheat
R. dominica	*JKI*	FRCCP (Laboratory)	Wheat
R. dominica	*QRD14*	QDAF (Laboratory)	Wheat
R. dominica	*GA6*	Greece	Wheat
O. surinamensis	*USDA*	CGAHR (Laboratory)	Oat flakes with brewer's yeast
O. surinamensis	*UTH*	LEAZ (Laboratory)	Oat flakes with brewer's yeast
O. surinamensis	*DD*	DD (Laboratory)	Oat flakes with brewer's yeast
O. surinamensis	*ASC11*	Greece	Oat flakes with brewer's yeast
O. surinamensis	*W1*	Germany	Oat flakes with brewer's yeast
C. ferrugineus	*USDA*	CGAHR (Laboratory)	Oat flakes with brewer's yeast and cracked wheat
C. ferrugineus	*IRTA*	IRTA (Laboratory)	Oat flakes with brewer's yeast and cracked wheat
L. serricorne	*USDA*	CGAHR (Laboratory)	Flour with brewer's wheat and cracked wheat
L. serricorne	*JKI*	FRCCP (Laboratory)	Flour with brewer's wheat and cracked wheat
L. serricorne	*DD*	DD (Laboratory)	Flour with brewer's wheat and cracked wheat
L. serricorne	*E1*	Malaysia	Flour with brewer's wheat and cracked wheat
C. maculatus	*USDA*	CGAHR (Laboratory)	Cowpeas
A. obtectus	*USDA*	CGAHR (Laboratory)	Beans
O. mercator	*USDA*	CGAHR (Laboratory)	Oat flakes with brewer's yeast
T. variabile	*USDA*	CGAHR (Laboratory)	Dog food with oat flakes and cracked wheat

CGAHR: Center for Grain and Animal Health Research, USA, Manhattan; LEAZ: Laboratory of Entomology and Agricultural Zoology, Greece, Volos; QDAF: Queensland Department of Agriculture and Fisheries, Mareeba, Australia; FRCCP: Federal Research Center of Cultivated Plants, Berlin, Germany; IRTA: Institute for Food and Agricultural Research and Technology, Catalonia Spain; DD: Detia Degesch GmbH, Laudenbach, Germany.

and require specialized training and equipment. At the same time, these protocols have been designed for specific stored-product insects and thus, the approaches are not necessarily transferable to other species. As a result, many research groups throughout the globe evaluate phosphine resistance based on the "classic" Food and Agriculture Organization of the United Nations (FAO) evaluation protocol (Benhalima et al., 2004; Opit et al., 2012; Holloway et al., 2016; Afful et al., 2017), which typically involves exposure of insects in vials or jars that contain 30 ppm phosphine for 20 h (h) (FAO, 1975). This test, which was originally designed during the seventies, has been also thoroughly modified towards the quantification of resistance by several research groups (Yongsheng et al., 1999; Cato et al., 2017; Afful et al., 2017). Other researchers use dose response bioassays, on which insects are usually exposed for 3 d at different concentrations of the gas (Mills, 1986; Chaudhry, 2000). Despite modifications, the FAO and the dose response tests are also laborious, since they require special equipment (such as a Gas Chromatography to measure phosphine concentration) and specialized personnel, and, at the same time, require several days to yield results.

Nayak et al. (2013) presented a rapid bioassay test, which examines knockdown of the exposed insects and can differentiate weak and strong resistance to phosphine. This test is much shorter than the previous ones (FAO protocol), as it can provide reliable conclusions after approx. 5 h of exposure, and usually sooner in the case of susceptible or weak resistant strains. Another quick test is the Detia Degesch Phosphine Tolerance Test Kit (DDPTTK) (Detia Degesch GmbH, Germany), with which adults to be tested are exposed to a high gas concentration (i.e., 3000 ppm) usually for less

Table 2
Percentage (%) ± SE of knocked down adults after exposure to 30 ppm of phosphine for 20 h, for the some of the tested populations.

Populations	% of knocked down adults
ASC11 S. oryzae	9.4 ± 5.8[a]
ASC11 O. surinamensis	0.0 ± 0.0
GA6 R. dominica	0.0 ± 0.0
UTH S. oryzae	100.0 ± 0.0[a]
UTH S. granarius	100.0 ± 0.0
UTH O. surinamensis	100.0 ± 0.0[a]
UTH R. dominica	100.0 ± 0.0
UTH T. confusum	100.0 ± 0.0
UTH T. castaneum	100.0 ± 0.0

[a] From Agrafioti and Athanassiou (2018).

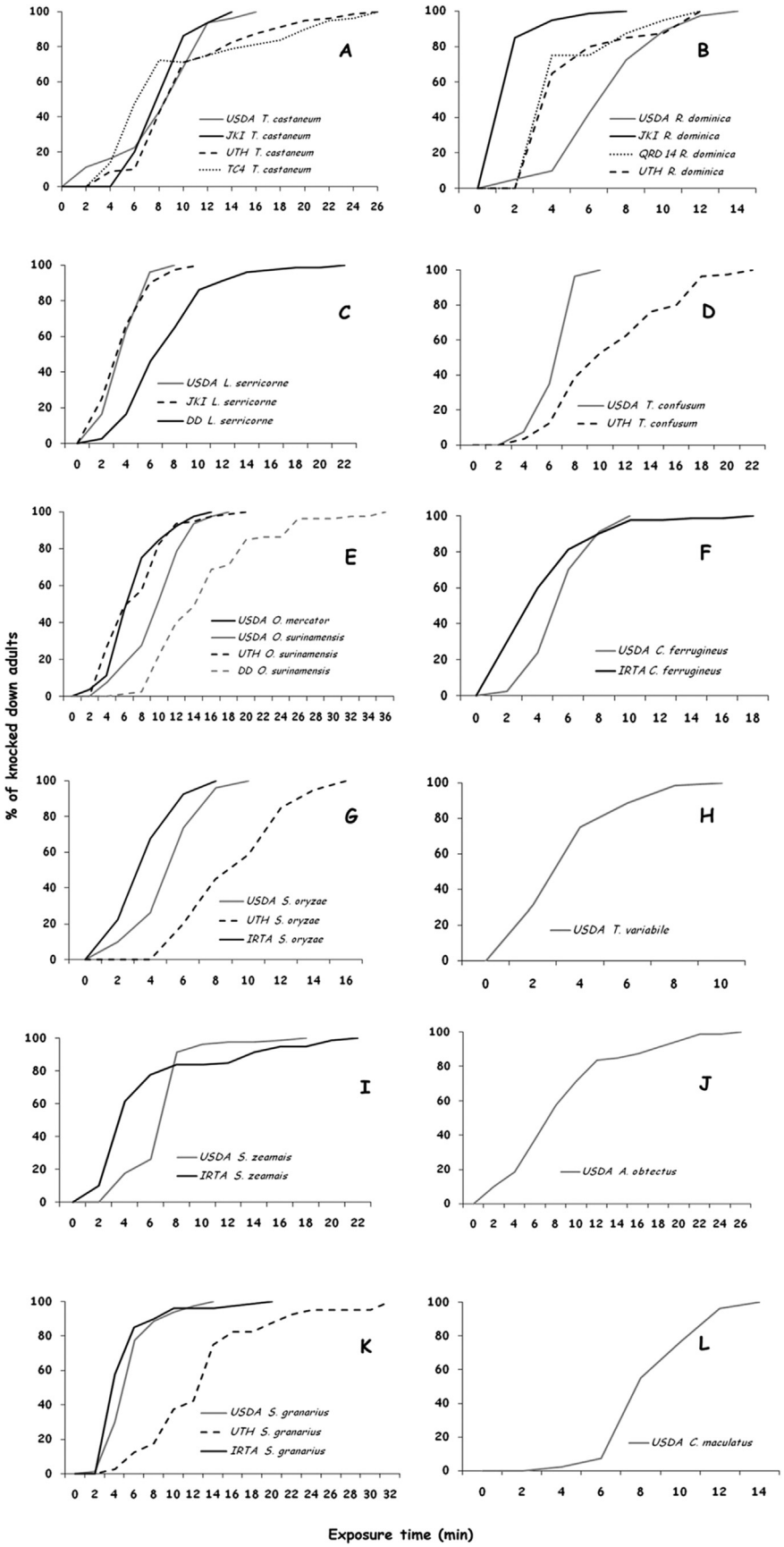

Fig. 1. Percentage (%) of knocked down adults of different laboratory strains of stored product beetle species, after exposure to phosphine at 1000 ppm for different observation intervals (in min): *T. castaneum* (A), *R. dominica* (B), *L. serricorne* (C), *T. confusum* (D), *O. surinamensis* (E), *C. ferrugineus* (F), *S. oryzae* (G), *T. variabile* (H), *S. zeamais* (I), *A. obtectus* (J), *S. granarius* (K), and *C. maculatus* (L).

than 15 min, and uses knockdown as indicator of resistance (Steuerwald et al., 2006; Athanassiou et al., 2019). This test can be operated on site by non-specialized personnel (with no specific license) using inexpensive equipment, and can rapidly provide some initial information on the resistance status (Aulicky et al., 2015). The DDPTTK tests, and also the test of Nayak et al. (2013), use deviations from normal mobility (walking behavior) of the exposed adults as indicators of susceptibility to the fumigant.

Insects affected by phosphine exhibit a range of responses from slightly less coordinated walking to complete knockdown and hence, their classification is observer-depended, and as such can be biased. In general, the "speed to knockdown" has been utilized as an indicator of resistance (Nayak et al., 2013; Aulicky et al., 2015). Classifying beetles as either "moving normally" or "not moving normally", with the latter group containing all individuals along the continuum of knockdown to death, would be a simpler approach. Measuring the time to transition from moving normally to being knocked down may be less observer-depended and more accurate indicator of response to fumigation in a wider range of cases.

Although quantification of deviations from normal mobility is valuable for assessing resistance levels, the actual critical threshold that differentiates resistant from susceptible populations is poorly understood. Reichmuth (1991) suggested that for *R. dominica*, resistance can be estimated by exposing adults of this species at 1000–3000 ppm for 30 min, and that knockdown can be used as indicator of resistance. However, knockdown in susceptible populations is also related with lower concentrations (Winks, 1985; Waterford and Winks, 1994). While there have been numerous studies that have evaluated resistance to phosphine, the majority of these studies are based on the comparison of field collected populations with a population classified as susceptible. In this context, often resistance of a given population is expressed as a function of the performance of the susceptible population or performance to a population classified as resistant. Given the limited fitness costs for resistance to phosphine that have been reported, it is often difficult to identify "completely" susceptible populations. Hence, often the classification of populations as resistant or susceptible is vague, since in most studies only one population of each type is used, and the results might have been different if different reference populations are used. Moreover, these rapid tests (i.e., by exposing of adults to phosphine only for some minutes) have not been evaluated in detail for the majority of the key stored-product insect

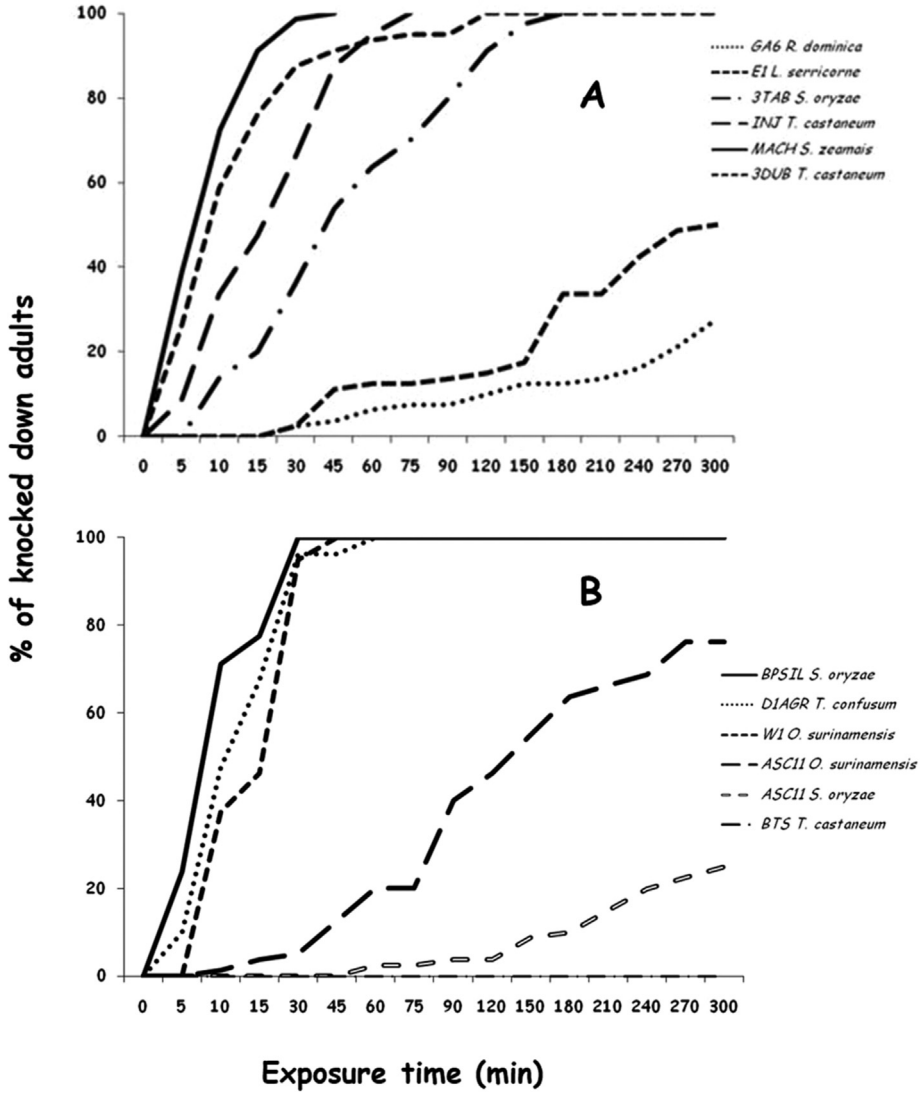

Fig. 2. Percentage (%) of knocked down adults of different field strains of stored product beetle species, after exposure to phosphine at 1000 ppm for different observation intervals (in min).

species.

In the present study, we used the DDPTTK to estimate susceptibility to phosphine by testing multiple susceptible populations of different major stored-product insect species, as well as multiple field populations. For this purpose, we examined several laboratory and field populations obtained from different laboratories. Moreover, we defined critical thresholds for the populations tested, in order to form a rapid bioassay tool for the characterization of phosphine resistance. Hence, in the present work we attempted to revise some of the critical times-to-knockdown that are labeled on the DDPTTK, and also to provide data for additional species, in an effort to contribute further in the adoption of a rapid diagnostic test for resistance to phosphine.

2. Materials and methods

2.1. Insects

The laboratory and field populations that were used in the tests corresponded to thirteen stored product beetle species, and were obtained from different laboratories in different countries. The populations used were laboratory populations from USA, Greece, Australia, Germany and Spain. Also, there were some field populations that have been collected from different facilities between 2013 and 2017. The origin of the populations tested and the rearing media of each species are shown on Table 1. All laboratory populations were susceptible to phosphine according to preliminary tests. All species were reared at laboratory conditions of 25 °C, and 65% relative humidity (r.h.). Adults, of mixed sex and age, were used in the bioassays.

2.2. Screening through the FAO protocol

The protocol used to evaluate resistance to phosphine was the FAO bioassay as described by FAO (1975). This protocol was followed for some of the populations tested (Table 2), as suggested by Agrafioti and Athanassiou (2018). The tests were carried out with 20 adults of the tested populations in a 1 l glass jar that contained 30 ppm of phosphine for 20 h. These trials were carried out at 25 °C, 65% r.h. and continuous darkness. After the termination of this interval, knocked down adults were recorded, while in most of the cases, knocked down insects were dead. For these tests, there were three replicates with three subreplicates per population.

Table 3
Probit Analysis for KDt_{50}, KDt_{95} and KDt_{99} (confidence intervals) of adults after exposure to 1000 ppm concentration of phosphine for the insect populations tested, expressed as minutes to knockdown, using the DDPTTK.

Populations (code, species)	KDt_{50}	KDt_{95}	KDt_{99}	Slope ± SE	X^2	*P*
USDA T. castaneum	6.8 (5.8–7.8)	17.7 (14.3–24.7)	26.3 (19.8–41.7)	3.9 ± 0.2	109.1	<0.01
UTH T. castaneum	8.8 (8.0–9.5)	19.3 (17.4–22.0)	16.8 (23.4–32.1)	4.8 ± 0.2	97.2	<0.01
TC4 T. castaneum	7.2 (5.7–8.5)	22.9 (18.3–32.4)	37.0 (27.2–61.2)	3.2 ± 0.1	271.2	<0.01
JKI T. castaneum	7.6 (7.1–8.1)	11.7 (10.7–13.2)	14.0 (12.5–16.5)	8.9 ± 0.7	47.3	<0.01
BTS T. castaneum	[b]	[b]	[b]	[b]	[b]	[b]
INJ T. castaneum	15.9 (12.4–149.5)	60.1 (46.1–87.8)	104.4 (73.9–175.6)	2.8 ± 0.1	251.0	<0.01
3DUB T. castaneum	8.5 (6.6–10.4)	56.6 (46.0–73.4)	124.0 (92–181.5)	2.0 ± 0.1	77.9	0.04
USDA T. confusum	6.0 (5.6–6.3)	8.5 (7.9–9.4)	9.8 (8.9–11.3)	10.7 ± 0.9	35.2	0.10
UTH T. confusum	9.6 (8.5–10.7)	19.9 (17.1–25.3)	26.9 (21.9–37.5)	5.2 ± 0.3	188.0	<0.01
D1AGR T. confusum	10.9 (9.9–12.6)	31.4 (25.4–42.8)	48.8 (36.9–74.3)	3.5 ± 0.2	123.0	<0.01
USDA S. oryzae	4.3 (3.8–4.8)	8.6 (7.4–1.6)	11.5 (9.5–15.3)	5.5 ± 0.4	78.3	<0.01
UTH S. oryzae	8.4 (8.0–8.8)	14.4 (13.4–15.8)	18.0 (16.4–20.3)	7.0 ± 0.5	25.7	0.69
IRTA S. oryzae	2.9 (2.5–3.3)	6.5 (5.5–8.4)	9.0 (7.2–12.9)	4.8 ± 0.4	23.3	0.05
3TAB *S. oryzae*	37.1 (30.4–44.2)	154.1 (121.8–211.4)	277.8 (203–429)	2.6 ± 0.1	212.0	<0.01
BPSIL S. oryzae	7.8 (6.8–8.6)	21.7 (18.4–27.0)	33.1 (26.6–45.2)	3.7 ± 0.3	57.0	0.49
ASC11 S. oryzae	592[a]	3110.2[a]	6184[a]	2.2 ± 0.2	98.0	0.98
USDA S. granarius	4.8 (4.1–5.7)	9.5 (8.2–11.6)	12.6 (10.4–16.7)	5.5 ± 0.4	100.1	<0.01
UTH S. granarius	11.4 (10.5–12.2)	25.3 (23.0–28.4)	35.2 (31.0–41.4)	4.7 ± 0.3	14.7	0.99
IRTA S. granarius	4.1 (3.4–4.7)	10.0 (8.6–12.2)	14.5 (11.8–19.3)	4.2 ± 0.2	96.3	<0.01
USDA S. zeamais	6.0 (5.5–6.6)	10.8 (9.6–12.6)	13.7 (11.8–16.9)	6.6 ± 0.4	79.5	<0.01
IRTA S. zeamais	4.0 (2.5–5.2)	15.2 (11.3–25.5)	26.5 (17.6–58.6)	2.8 ± 0.1	292.3	<0.01
MACH S. zeamais	6.2 (4.9–7.4)	19.1 (15.2–27.7)	30.4 (22.2–52.5)	3.3 ± 0.3	107.0	<0.01
USDA R. dominica	6.0 (5.3–6.6)	12.0 (10.4–14.6)	16.0 (13.3–21)	5.4 ± 0.3	83.8	<0.01
UTH R. dominica	4.1 (3.6–4.6)	9.9 (8.6–12.0)	12.3 (10.4–15.6)	4.3 ± 0.3	33.5	0.05
JKI R. dominica	0.8 (0.2–1.2)	3.5 (2.8–4.9)	6.4 (4.6–14.3)	2.6 ± 0.6	10.9	0.68
QRD14 R. dominica	3.9 (2.8–4.8)	8.8 (6.9–14.5)	12.4 (8.9–25.6)	4.6 ± 0.5	29.0	0.01
GA6 R. dominica	1098[a]	18970[a]	61765[a]	1.3 ± 0.1	64.0	0.27
USDA O. surinamensis	8.7 (8.1–9.4)	16.3 (14.6–19.0)	21.1 (18.2–26.0)	6.0 ± 0.3	75.1	<0.01
UTH O. surinamensis	6.1 (5.5–6.8)	14.0 (12.3–16.5)	19.6 (16.5–24.8)	4.6 ± 0.2	81.1	<0.01
DD O. surinamensis	14.0 (12.7–15.2)	26.9 (24.0–31.4)	35.2 (30.3–43.7)	5.8 ± 0.2	325.8	<0.01
ASC11 O. surinamensis	136.6 (115–164)	749.4 (515–1337)	1517 (922–3315)	2.2 ± 0.1	208.0	<0.01
W1 O. surinamensis	13.7 (11.8–15.7)	30.3 (24.7–42.1)	42.1 (32.3–65.4)	4.7 ± 0.4	148.0	<0.01
USDA C. ferrugineus	4.9 (4.3–5.3)	8.6 (7.7–10.2)	10.9 (9.4–13.9)	6.6 ± 0.5	53.9	<0.01
IRTA C. ferrugineus	3.0 (2.7–3.4)	9.7 (8.6–11.3)	15.7 (13.2–19.5)	3.2 ± 0.2	41.4	0.17
USDA L. serricorne	3.1 (2.9–3.4)	6.1 (5.5–7.0)	8.1 (7.1–9.7)	5.6 ± 0.4	13.7	0.95
JKI L. serricorne	3.0 (2.4–3.4)	7.1 (5.8–9.6)	10.2 (7.9–15.8)	4.3 ± 0.3	43.0	<0.01
DD L. serricorne	6.2 (5.4–6.9)	13.8 (12.0–16.7)	19.2 (16.0–25.0)	4.7 ± 0.2	123.7	<0.01
E1 L. serricorne	325[a]	2580[a]	6080[a]	1.8 ± 0.1	268.0	<0.01
USDA C. maculatus	7.9 (7.3–8.5)	12.1 (10.9–14.0)	14.4 (12.7–17.6)	9.0 ± 0.7	69.1	<0.01
USDA A. obtectus	6.6 (6.1–7.1)	20.3 (18.4–22.8)	32.3 (28.2–38.1)	3.3 ± 0.1	47.4	0.57
USDA O. mercator	6.0 (5.3–6.7)	12.8 (11.1–15.8)	17.5 (14.4–23.3)	5.0 ± 3.4	82.6	<0.01
USDA T. variabile	2.7 (2.3–2.9)	6.6 (5.9–7.7)	9.6 (8.2–11.9)	4.2 ± 0.3	34.2	0.45

[a] Could not estimate confidence intervals.
[b] Could not estimate knockdown time.

2.3. Bioassays with the DDPTTK protocol

We used the standard DDPTTK and methodology (Steuerwald et al., 2006), at two concentrations of phosphine, 1000 and 3000 ppm, as modified by Athanassiou et al. (2019), with some modifications. Twenty adults of the tested populations (separate sets of adults in each replicate) were placed in a plastic 100 ml syringe from the kit. The phosphine gas was generated by adding two kit tablets (designed exclusively for the kit) to 50 ml of water, within a plastic canister of 5 l capacity. The concentration of the gas inside the canister was determined by using glass tubes (Draeger25A, Draeger Safety AG & Co., Germany) (Steuerwald et al., 2006; Athanassiou et al., 2019). Then, a specific gas quantity was removed from the canister with the syringe, to achieve a concentration of either 1000 or 3000 ppm in the syringe in a total air volume of 100 ml. Additional syringes with insects that contained only air, were used as controls. The mobility of adults was observed every 2 min. They were classified into two categories: a) individuals that moved normally, i.e., capable of coordinated movement and b) individuals that were immobilized (knocked down), i.e., not capable of coordinated movement. The observations of the insects in a syringe were terminated 2 min after the time that all individuals were classified as not normally moving. There were two replicates for each concentration (1000 and 3000 ppm), species and population, (2 x 2 = 4 syringes total), by preparing new gas canister for each block (total 2 canisters for each species, concentration and population).

2.4. Data analysis

The data for the insects that had been exposed to air alone (control syringes) showed no knockdown, so they were not included in the analysis. For the DDPTTK counts, separately for each concentration, species and population, the data were analyzed by using Probit Analysis to estimate knockdown time, i.e., KDt_{50}, KDt_{95} and KDt_{99}. For all cases, we applied Regression Probit Analysis by using the SPSS software (IBM Corp., 2016).

3. Results

3.1. Results with the FAO protocol

From the populations tested, all laboratory populations were knocked down after the termination of the 20 h exposure (Table 2). In contrast, for the field populations tested, all *ASC11 O. surinamensis* and *GA6 R. dominica* adults were capable of coordinated movement.

Table 4
Probit Analysis for KDt_{50}, KDt_{95} and KDt_{99} (confidence intervals) of adults after exposure to 3000 ppm concentration of phosphine for the populations tested, expressed as minutes to knockdown, using the DDPTTK.

Populations (code, species)	KDt_{50}	KDt_{95}	KDt_{99}	Slope ± SE	X^2	*P*
USDA T. castaneum	3.2 (3.0–3.5)	5.9 (5.4–6.8)	7.7 (6.7–9.1)	6.2 ± 0.5	22.0	0.42
UTH T. castaneum	2.0 (0.6–3.1)	7.0 (4.8–21.5)	11.7 (7.0–76.6)	3.0 ± 0.3	106.2	<0.01
TC4 T. castaneum	0.8 (0.09–1.3)	2.5 (2.0–3.8)	3.9 (3.0–13.9)	3.5 ± 1.2	14.0	0.17
JKI T. castaneum	1.1 (0.5–1.4)	3.2 (2.7–4.2)	4.9 (3.8–8.6)	3.5 ± 0.7	22.6	0.22
BTS T. castaneum	679[a]	2713[a]	4815[a]	2.7 ± 0.4	23.4	0.99
INJ T. castaneum	2.8 (1.5–3.8)	9.4 (7.7–13.2)	15.3 (11.4–29.5)	3.2 ± 0.6	20.9	0.99
3DUB T. castaneum	9.0 (5.6–12.7)	95.3 (68.7–149)	252.0 (158–507)	1.6 ± 0.1	176.0	<0.01
USDA T. confusum	4.5 (3.9–5.0)	8.5 (7.3–10.6)	11.0 (9.1–14.9)	6.0 ± 0.4	74.4	<0.01
UTH T. confusum	2.8 (0.9–4.2)	7.2 (4.6–8.8)	10.7 (5.9–30.3)	4.0 ± 0.4	88.9	<0.01
D1AGR T. confusum	4.1 (2.6–5.2)	14.1 (11.0–22.1)	23.5 (16.5–48.9)	3.0 ± 0.4	94.7	<0.01
USDA S. oryzae	2.9 (2.6–3.1)	4.9 (4.4–5.6)	6.1 (5.4–7.3)	7.1 ± 0.6	11.0	0.89
UTH S. oryzae	2.0 (1.6–2.3)	5.0 (4.2–6.5)	7.3 (5.8–10.9)	4.1 ± 0.4	25.0	0.12
IRTA S. oryzae	0.9[a]	1.7[a]	2.3[a]	5.6 ± 8.6	2.0	0.99
3TAB *S. oryzae*	16.2 (14.3–18.2)	57.1 (48.5–69.9)	96.2 (77.7–126.1)	3.0 ± 0.1	74.5	0.07
BPSIL S. oryzae	2.3 (0.8–3.4)	8.4 (6.8–12.0)	14.5 (10.7–31.0)	2.9 ± 0.7	21.9	0.99
ASC11 S. oryzae	221.0 (205.0–242.0)	521 (435–677)	743.0 (588–1049)	4.4 ± 0.3	108.0	<0.01
USDA S. granarius	2.2 (2.0–2.4)	3.6 (3.2–4.5)	4.5 (3.8–5.9)	7.5 ± 1.0	14.4	0.95
UTH S. granarius	3.9 (3.3–4.3)	9.1 (7.9–11.1)	13.1 (10.8–17.1)	4.4 ± 0.4	8.8	0.75
IRTA S. granarius	0.8 (0.4–1.3)	5.4 (4.4–7.2)	11.7 (8.5–20.2)	2.0 ± 0.3	15.8	0.94
USDA S. zeamais	4.6 (4.0–5.0)	7.3 (6.5–9.0)	8.9 (7.6–11.9)	8.0 ± 0.7	72.5	<0.01
IRTA S. zeamais	0.7[a]	1.5[a]	2.0[a]	5.4 ± 12.7	0.9	0.82
MACH S. zeamais	0.036[a]	0.9[a]	3[a]	1.3 ± 1.7	9.9	0.99
USDA R. dominica	2.8 (2.5–3.1)	6.6 (5.9–7.7)	9.4 (8.0–11.7)	4.4 ± 3.9	24.9	0.29
UTH R. dominica	2.7 (2.3–3.1)	5.6 (4.7–7.2)	7.5 (6.1–10.7)	5.3 ± 0.4	37.1	<0.01
JKI R. dominica	2.3 (0.6–3.4)	6.5 (4.3–37.6)	9.9 (5.8–161.4)	3.7 ± 0.3	1941.2	<0.01
QRD14 R. dominica	1.9[a]	7.2[a]	12.5[a]	2.8 ± 0.6	107.6	<0.01
GA6 R. dominica	65.6 (58.5–72.8)	213.0 (182.5–260)	347.0 (282–454)	3.2 ± 0.1	116	<0.01
USDA O. surinamensis	4.7 (4.3–5.1)	8.6 (7.7–9.9)	11.0 (9.6–13.3)	6.3 ± 0.4	36.9	0.07
UTH O. surinamensis	1.9 (1.5–2.2)	3.7 (3.1–5.2)	4.8 (3.8–8.0)	5.9 ± 0.8	29.6	0.04
DD O. surinamensis	0.8 (0.3–1.3)	5.3 (4.3–7.2)	11.3 (8.1–21.1)	2.0 ± 0.3	23.4	0.37
ASC11 O. surinamensis	68.4 (59.0–78.1)	352 (281–474)	696.0 (512–1051)	2.3 ± 0.1	127.9	<0.01
W1 O. surinamensis	5.0 (4.3–5.7)	11.8 (10.1–15.1)	16.8 (13.5–24.3)	4.4 ± 0.6	64.6	0.25
USDA C. ferrugineus	3.4 (3.2–3.7)	6.4 (5.8–7.2)	8.2 (7.3–9.7)	6.1 ± 0.5	20.2	0.78
IRTA C. ferrugineus	2.7 (2.0–3.3)	5.4 (4.2–8.8)	7.1 (5.36–14.3)	5.4 ± 0.5	97.3	<0.01
USDA L. serricorne	2.5 (2.3–2.7)	4.9 (4.3–5.7)	6.4 (5.5–8.0)	5.7 ± 0.5	7.4	0.99
JKI L. serricorne	2.1 (1.9–2.3)	3.5 (3.1–4.4)	4.4 (3.7–5.9)	7.2 ± 1.0	13.7	0.18
DD L. serricorne	1.5 (0.6–1.9)	3.0 (2.5–7.7)	4.0 (3.0–20.5)	5.5 ± 1.2	19.6	0.03
E1 L. serricorne	36.4 (31.7–41.1)	118 (100–144)	192.0 (156–252)	3.2 ± 0.1	117	<0.01
USDA C. maculatus	3.4 (3.1–3.7)	6.1 (5.4–7.1)	7.7 (6.7–9.4)	6.6 ± 0.5	34.0	0.14
USDA A. obtectus	3.0 (2.5–3.4)	8.3 (7.1–10.2)	12.7 (10.3–17.0)	3.7 ± 0.3	36.5	0.08
USDA O. mercator	4.1 (3.8–4.5)	9.5 (8.5–10.8)	13.3 (11.6–15.9)	4.6 ± 0.3	32.1	0.18
USDA T. variabile	1.2 (0.7–1.6)	4.4 (3.7–5.9)	7.5 (5.7–12.3)	2.9 ± 0.5	13.8	0.74

[a] Could not estimate the confidence intervals.

3.2. Exposure at 1000 ppm of phosphine

For laboratory susceptible populations, the shortest exposure time to 100% knockdown was recorded for *JKI R. dominica* (Fig. 1B) and *IRTA S. oryzae* (Fig. 1G), which did not exceed 8 min, and the longest time was recorded for *DD O. surinamensis*, which reached 36 min (Fig. 1E). For most of the tested populations, the time to 100% knockdown was less than 20 min (Fig. 1A–I, K, L). Moreover, with few exceptions, for the laboratory populations and species tested, the critical time that was required for 100% knockdown ranged between 10 and 16 min (Fig. 1A–H, K, L). For the field populations, the response times varied remarkably. For certain field populations (e.g., *MACH S. zeamais*, *BPSIL S. oryzae*, *D1AGR T. confusum*, *W1 O. surinamensis, 3DUB T. castaneum* and *INJ T. castaneum*), the intervals to 100% knockdown were similar or only slightly increased in comparison with those reported for the laboratory populations (Figs. 1 and 2). In contrast, for many field populations, the intervals were notably longer, and ranged between 150 and 300 min (e.g., 3TAB *S. oryzae*, *ASC11 S. oryzae*, *E1 L. serricorne*, *GA6 R. dominica*, *ASC11 O. surinamensis* and *BTS T. castaneum*) (Fig. 2A and B). For five of the field populations, complete knockdown did not occur even after the termination of the 300 min observation interval (Fig. 2A and B). These variations are also illustrated for the KDt values, where, in general, KDt_{95} values were close to the KDt_{99} ones, with the exception of *GA6 R. dominica*, *ASC11 O. surinamensis* and *ASC11 S. oryzae* (Table 3). For the laboratory populations, KDt_{95} values ranged between 3.5 and 22.9 min.

3.3. Exposure at 3000 ppm of phosphine

At this phosphine concentration, the time to reach 100% knockdown was notably reduced in comparison with 1000 ppm (Table 4). For the laboratory populations, the exposure intervals ranged between 2 and 14 min, with the longest intervals being recorded for *UTH S. granarius* (Fig. 3K), *USDA A. obtectus* (Fig. 3J) and *DD O. surinamensis* (Fig. 3E). When more than one laboratory population was available per species, the response generally did not exceed 12 min to 100% knockdown, with the exception of *UTH S. granarius* (Fig. 3K). For most of the tested populations, the time to 100% knockdown was less than 10 min (Fig. 1A–I, K, L). At the same time, for some of the field populations (e.g., *MACH S. zeamais*, *D1AGR T. confusum* and *INJ T. castaneum*), the exposure intervals that were required for 100% knockdown were comparable to those recorded for the laboratory populations (Figs. 3 and 4). For example, *MACH S. zeamais*, *D1AGR T. confusum* and *INJ T. castaneum* required 10, 15, and 15 min in order to reach 100% knockdown, respectively (Fig. 4A and B). In contrast, for the other field populations, the intervals to achieve 100% knockdown were much longer, and reached 300 min (Fig. 4A and B). Moreover, 100% knockdown was not achieved in the case of two populations, *ASC11 S. oryzae* and *BTS T. castaneum* (Fig. 4B), as compared to five populations at 1000 ppm (Fig. 2A and B). Variation in KDt values at 3000 ppm was lower than the respective values at 1000 ppm, especially for the laboratory populations (Tables 3 and 4).

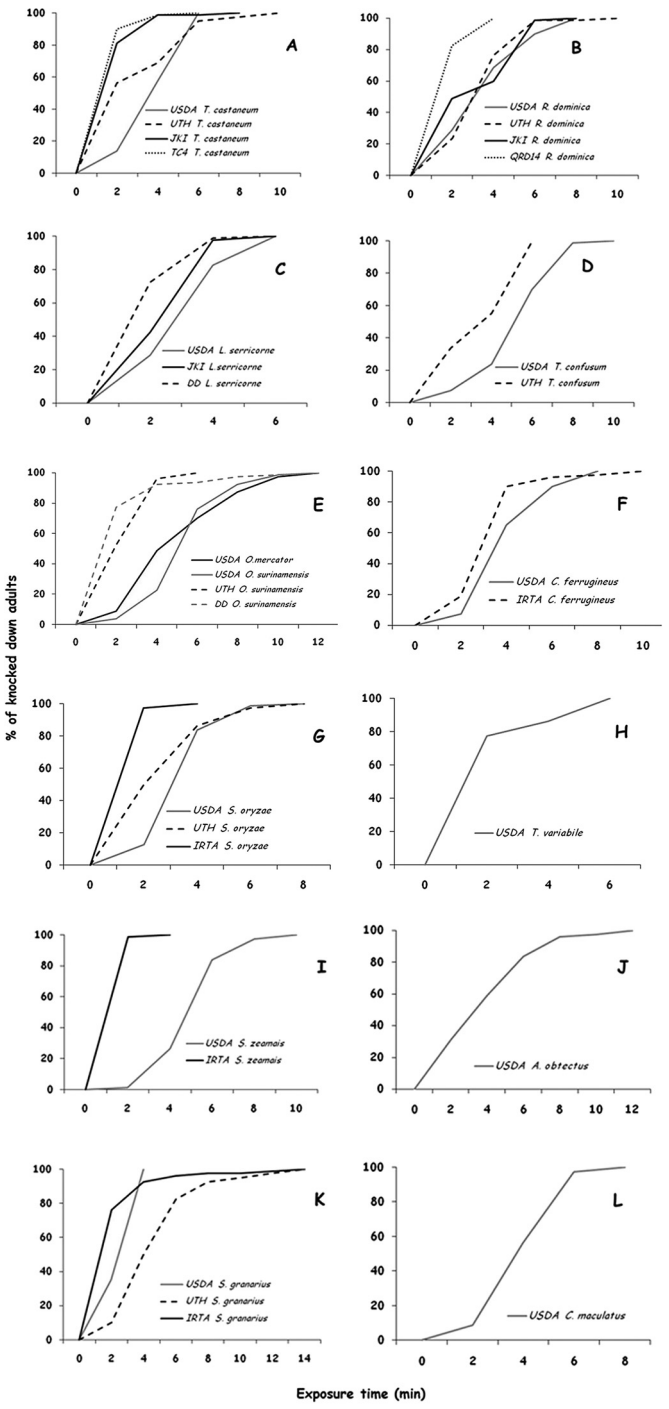

Fig. 3. Percentage (%) of knocked down adults of different laboratory strains of stored product beetle species, after exposure to phosphine at 3000 ppm for different observation intervals (in min): *T. castaneum* (A), *R. dominica* (B), *L. serricorne* (C), *T. confusum* (D), *O. surinamensis* (E), *C. ferrugineus* (F), *S. oryzae* (G), *T. variabile* (H), *S. zeamais* (I), *A. obtectus* (J), *S. granarius* (K), and *C. maculatus* (L).

4. Discussion

Immobilization (knockdown) of stored product insects after relatively short exposures to phosphine has long been investigated as a potential indicator of resistance, but the results so far are rather inconclusive (Winks, 1982, 1984; 1985; Reichmuth, 1991; Bell et al., 1994; Cao and Wang, 2001; Nayak et al., 2013; Cato, 2015). Nayak et al. (2013) tested *C. ferrugineus* populations from Australia and they found that the time-to-knockdown differed remarkably among populations that were susceptible, weakly resistant and strongly resistant to phosphine. In that study, the authors indicated that strong resistance to phosphine could be differentiated from either weak resistance or susceptibility, if the exposed insects are not knocked down after 5 h and 15 min at 2 mg/l of phosphine, respectively. Aulicky et al. (2015) reported that the time-to-knockdown of one laboratory and one field *T. confusum* strain, after using DDPTTK, differ remarkably. Cao and Wang (2001) designed a similar rapid bioassay, which could differentiate

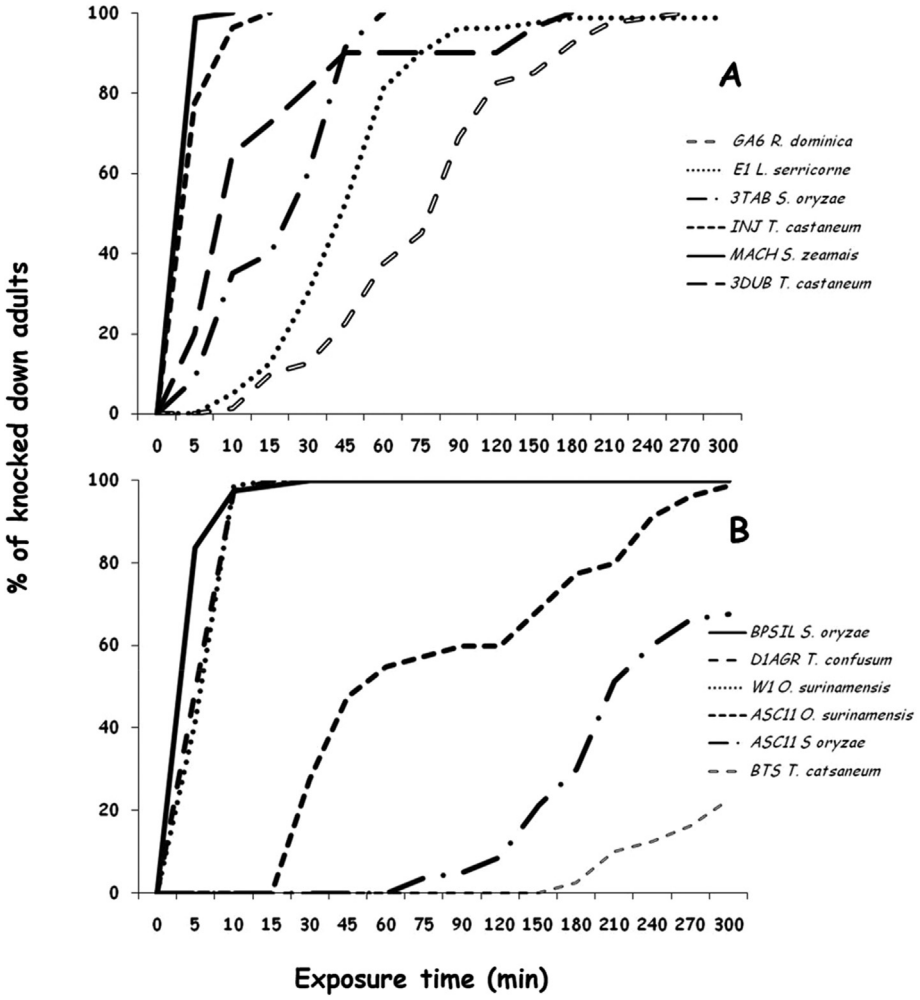

Fig. 4. Percentage (%) of knocked down adults of different field strains of stored product beetle species, after exposure to phosphine at 3000 ppm for different observation intervals (in min).

resistance status of a given insect strain after 30 min. Cato (2015) also found that adult knockdown could be quantified in terms of resistance characterization of *T. castaneum*. Athanassiou et al. (2019), by using the DDPTTK for one phosphine-susceptible and one phosphine-resistant population of *T. castaneum* found that the speed to knockdown was related with recovery patterns at certain post-exposure periods. The test that we described here has been designed under this principle, as it minimizes the amount of subjective observations since the adults are classified as "moving normally" and "not moving normally". During the tests, we observed that the initial deviation from normal movement, rapidly transitioned to knockdown. Interestingly, for the populations that have been evaluated through the FAO protocol here, the results with the DDPTTK showed similar results, in terms of the characterization of the resistance to phosphine of these populations. Additional experimental work is needed to compare these tests for a wider range of species and populations.

Based on the DDPTTK at 3000 ppm, Steuerwald et al. (2006) determined the "critical" intervals beyond which the tested populations should be characterized as tolerant/resistant as 11, 13, 12, 8, 8 and 15 min, for *O. surinamensis*, *C. ferrugineus*, *S. granarius*, *T. castaneum*, *L. serricorne* and *A. obtectus*, respectively. Our results (based on 3000 ppm) for the laboratory populations tested here suggest that, these "critical" times for the characterization of resistance should be slightly modified. Specifically, for the species mentioned above, we propose that the corresponding times should be between 6 and 14 min, based on KDt_{99} values for 3000 ppm or the data presented on Fig. 3 for the same concentration. Based on this, and using the critical times indicated above, some of the field populations can be classified as resistant. It should be noted, however, that the DDPTTK can be used to distinguish susceptible from resistant populations, but, at least based on our protocol, cannot be used to separate weak from strong resistance. So far, the test that has been proposed by Nayak et al. (2013) is the only quick test that can diagnose and separate weak from strong resistance.

During our tests, we observed that, occasionally, out of the 20 individuals which had been placed in the syringe, there was a small number of individuals, corresponding to 5% of the total (usually one adult), that were active for considerably longer intervals. This pattern had a certain effect the calculation of the KDt values, and this is why we propose critical times that were based on our estimated KDt_{95} values. At the same time, we consider that selecting 3000 ppm to carry out the DDPTTK is more accurate than tests based on 1000 ppm, as the variation among individuals that had been exposed in syringe was much greater at 1000 ppm than at 3000 ppm. For example, for *T. castaneum* populations, KDt_{50} values were considerably lower at 3000 ppm than at 1000 ppm. At the same time, at 3000 ppm the data were more uniform, and the

overall test lasted for a considerably shorter interval.

Winks and Waterford (1986) noted that, in the *T. castaneum* populations tested in their bioassays, phosphine-induced knockdown was not connected with resistance to phosphine. Therefore, in any effort to design resistance diagnostics that can be widely adopted, terminology clarifications are essential. Our aim was to draw specific critical times based on populations that are characterized as susceptible to phosphine, and not to quantify and categorize the "gray area" beyond these times. Still, it remains unclear if the insects that are knocked down during the short exposure of the DDPTTK test, will recover after their removal from the treated substrate. Previous results for *T. castaneum* clearly suggest that recovery patterns after certain exposure intervals to DDPTTK is related with resistance.

As many industries and fumigators do not have access to specialized laboratories, this kit is an easy-to-use and observer subjective solution to have a first indication of tolerance/resistance to phosphine levels at a location. We suggest that the test can be operated at 3000 ppm, and that the critical intervals to characterize reduced susceptibility range between the intervals indicated above, depending on the target species. Obviously, accurate identification of the species that are to be tested is the most essential parameter in using DDPTTK as an evaluation tool.

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