Research Article

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Scaling recovery of susceptible and resistant stored product insects after short exposures to phosphine by using automated video-tracking software

Paraskevi Agrafioti,[a*] Daniel L Brabec,[b] William R Morrison III,[b] James F Campbell[b] and Christos G Athanassiou[a,b,c]

Abstract

BACKGROUND: Phosphine-susceptible or resistant populations of *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) adults were exposed to 0 (control), 1000 and 3000 ppm of phosphine for 15 or 90 min, to estimate behavioral and mobility responses after exposure to phosphine. Knockdown of the exposed individuals after exposure was recorded visually. The total distance moved and velocity of movement were assessed immediately after exposure to phosphine, 2 or 24 h later using a camera coupled with automated video tracking software (i.e. Ethovision®).

RESULTS: For both species tested, the highest percentage of dead adults was noted at the highest concentration (3000 ppm) for both exposure times. For *T. castaneum*, total distance moved and velocity decreased as the concentration increased for the susceptible population, whereas there was significant variation among individuals in the resistant population. For *R. dominica*, the distance moved was reduced at the highest concentrations. Individuals of *R. dominica* moved less than those of *T. castaneum* and there were significant differences in mobility between susceptible and resistant populations for both species tested. Recovery was much faster in the case of the resistant populations.

CONCLUSIONS: Changes in movement parameters can be further exploited in assessing the efficacy of different management tactics, such as trapping and sampling. Automated video tracking systems such as Ethovision® can be used to track and record insect behavioral response, providing a more objective measure of insecticide efficacy than visual categorizations. These data shed light on insect mobility and behavioral responses to fumigation treatments in relation to resistance.

Keywords: Ethovision®; resistance; behavior; sublethal effects; post-harvest insects

1 INTRODUCTION

Phosphine is currently one of the main fumigants applied for the control of stored product insects in a wide range of durable commodities, such as dried fruits, legumes, tobacco and cereals.[1–4] Since methyl bromide was phased out,[5] the frequency of phosphine use for post-harvest treatments has increased significantly.[6,7] However, there are some limitations in the use of phosphine, such as the fact that phosphine is corrosive against certain metals, which may damage food facility equipment. In addition, phosphine is highly flammable at elevated concentrations, which may cause accidents during its application and disposal.[7] Improper and repeated use of phosphine gas has contributed to increasing resistance among a variety of species in many parts of the world.[7–12] Populations of more than 10 stored product insect species have been found with some level of resistance to phosphine.[2,7,10,12–16] For example, Gautam *et al.*[10] examined populations of the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) and the Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) collected from different locations in California and found that most of these populations were resistant to phosphine. A recent study by Aulicky *et al.*[16] indicated the first record of resistant populations of the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), adding one more species to the list of the ones with resistance. The genes responsible for the development of resistance to phosphine have been identified for some species, such as for *T. castaneum* and the lesser grain borer, *Rhyzopertha*

* *Correspondence to: P Agrafioti, Laboratory of Entomology and Agricultural Zoology, University of Thessaly, Phytokou str., 38446 Nea Ionia Magnessia, Greece, E-mail: agrafiot@agr.uth.gr*

a *Laboratory of Entomology and Agricultural Zoology, Department of Agriculture, Crop Production and Rural Environment, University of Thessaly, Volos, Greece*

b *Center for Grain and Animal Health Research, USDA, Agricultural Research Service, Manhattan, KS, USA*

c *Institute of Bio-Economy and Agri-Technology (iBO), Center for Research and Technology, Volos, Greece*

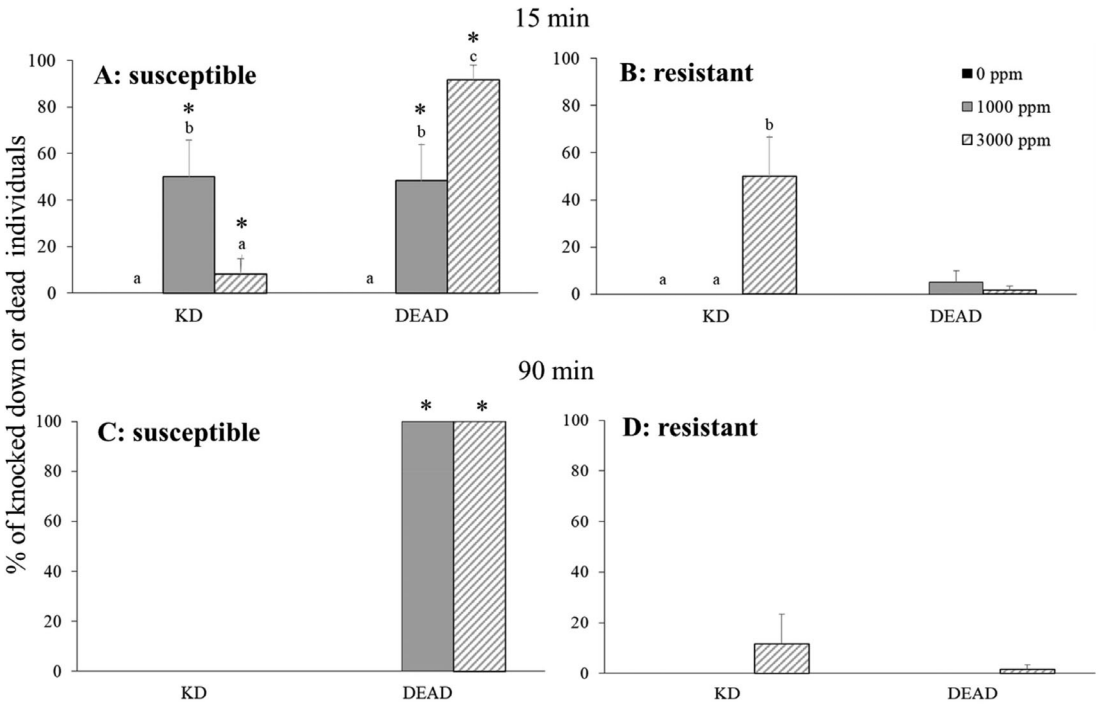

Figure 1. Percentage of knocked down (KD) or dead individuals of *Tribolium castaneum* that were exposed to 0 (control), 1000 and 3000 ppm for (A) 15 min in susceptible population, (B) 15 min in resistant population, (C) 90 min in susceptible population, (D) 90 min in resistant population. Within each population and exposure interval, separately for knocked down and dead individuals, means followed by the same lowercase letter do not differ significantly according to the Tukey–Kramer HSD test at $\alpha < 0.05$. Where no letter exists, no significant differences were noted. ANOVA parameters for knocked down individuals were for (A) $F = 7.27$, $P < 0.01$, for (B) $F = 8.92$, $P < 0.01$, and for dead individuals were for (A) $F = 22.09$, $P < 0.01$, in all cases $df = 2, 17$. Means with asterisks (*), obtained on susceptible populations, are significantly different from the respective means, obtained on resistant populations, for each exposure interval and concentration (1000 and 3000 ppm), according to Students' *t*-test at $\alpha < 0.05$. According to the *t*-test, the parameters for dead individuals at 1000 ppm were: for (A,B) $t = 2.64$, $P < 0.05$, for (C,D) $t = 2.99$, $P < 0.05$ and at 3000 ppm were: for (A,B) $t = 13.33$, $P < 0.01$, for (C,D) $t = 59.0$, $P < 0.01$. According to the t-test, the parameters for knocked down individuals at 1000 ppm were: for (A,B) $t = 3.14$, $P < 0.05$ and at 3000 ppm were: for (A,B) $t = -2.31$, $P < 0.05$, in all cases $df = 10$. For the rest of the combinations, *t*-test could not be performed.

dominica (F.) (Coleoptera: Bostrychidae).[7,17] These reports underline the importance of phosphine resistance among stored product insect species globally.

In detecting and evaluating resistance to phosphine, there are several protocols that have been developed and evaluated.[8,12,18–20] A recent study, Athanassiou *et al.*[19] examined the insect mobility patterns of phosphine-resistant and -susceptible adults of *T. castaneum* after exposure to phosphine at two concentrations (1000 and 3000 ppm). In that study, the authors found that quick immobilization leads to slow or no recovery, and delayed immobilization leads to quick recovery.[19] Thus, the use of behavioral indicators may be a quick method to reveal the occurrence of resistance, with two major behavioral diagnostics that have been taken into account up to now. The first is the immediate result of exposure, also termed 'immobilization', and refers to the immediate reaction of the exposed individuals during or right after the exposure.[9,12] The second is the delayed effect, also termed 'recovery', indicating the resumption of normal movement patterns at a certain post-exposure period.[19] The latter may reveal misleading behavioral patterns exhibited during exposure; if high initial immobilization is not always correlated with low mobility and recovery after exposure. For example, prior work with a population of *T. castaneum*, found that despite initial high immobilization, all individuals recovered 7 days later.[19]

A challenge with behaviorally-based methods to date is that quantification is relied on researcher observations and classifications and therefore vulnerable to observer-bias as well as variation in classification among observers. While behavioral states of full movement and no movement are quite easily classified, there is a grey area between these two extremes in which consistent classification is more challenging. Automated video-tracking systems coupled with software has been used for years to evaluate the movement of different stored product insect species and life stages and how movement changes in response to insecticide exposure.[21–26] For example, movement patterns of two psocid species (*Liposcelis bostrychophila* Badonnel and *Liposcelis entomophila* (Enderlein) (Psocoptera: Liposcelidae) varied on surfaces treated with different contact insecticides (*β*-cyfluthrin, chlorfenapyr and pyrethrins) and the reduced mobility of *L. bostrychophila* may be a contributing factor to its higher insecticide tolerance.[22] Arthur *et al.*[25] examined movement patterns of three stored-product beetle species, after exposure to a formulation of deltamethrin + methoprene + piperonyl butoxide by using tracking software, and found that species varied remarkably in their behavior after exposure. Important behavioral measures in prior studies have included total distance moved, velocity, angular velocity, and average turn angle. These behavioral changes can be parametrized further to quickly predict insecticidal efficacy, without needing to always conduct mortality-based bioassays.[25] Recently, Sakka *et al.*[27] calculated key behavioral parameters such as distance

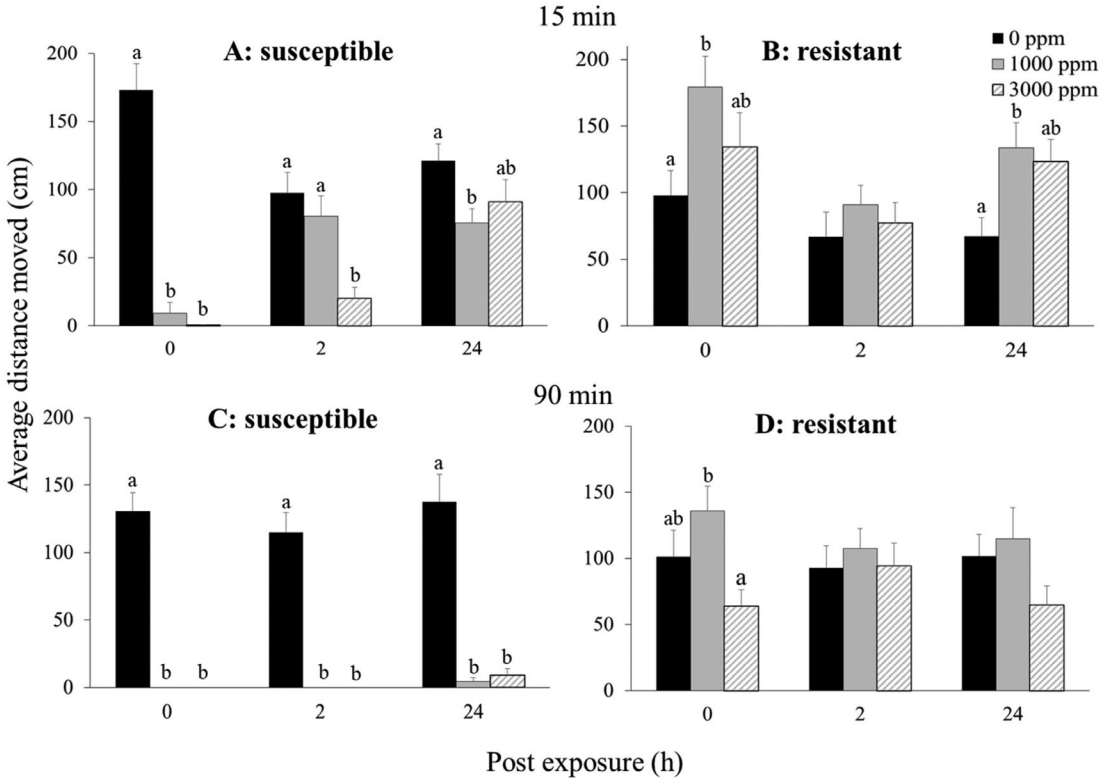

Figure 2. Average distance moved (cm) for *Tribolium castaneum* exposed to 0 (control), 1000 and 3000 ppm for (A) 15 min in susceptible population, (B) 15 min in resistant population, (C) 90 min in susceptible population, (D) 90 min in resistant population. Within each population and post exposure interval, means followed by the same lowercase letter do not differ significantly according to the Tukey–Kramer HSD test at $\alpha < 0.05$. Where no letters exist no significant differences were noted. ANOVA parameters for (A) 0-h F = 62.80, $P < 0.01$, 2-h F = 9.47, $P < 0.01$, 24-h F = 3.08, $P = 0.05$, for (B) 0-h F = 3.25, $P = 0.04$, 2-h F = 0.54, $P = 0.58$, 24-h F = 4.68, $P = 0.01$, for (C) 0-h F = 86.66, $P < 0.01$, 2-h F = 60.34, $P < 0.01$, 24-h F = 38.32, $P < 0.01$ and for (D) 0-h F = 4.30, $P < 0.01$, 2-h F = 0.23 $P = 0.79$, 24-h F = 1.82 $P = 0.17$, in all cases $df = 2, 53$.

moved and velocity for populations of *T. castaneum* and *R. dominica* that had different levels of phosphine resistance.

To our knowledge, video-based tracking has not been used to characterize sublethal effects of phosphine exposure on movement of stored product insect species with differential susceptibility to phosphine. We hypothesize that detailed measurements of movement parameters could be further exploited to characterize insect response to phosphine, an essential fumigant in the post-harvest supply chain around the world. The objective of the present study was to develop an experimental methodology for using Ethovision® to examine mobility parameters of populations of two cosmopolitan species (*T. castaneum* and *R. dominica*) that were known to be susceptible or resistant to phosphine at different post-exposure intervals.

2 MATERIALS AND METHODS

2.1 Tested insects

Two populations of *T. castaneum* and *R. dominica* were used, one susceptible and one resistant to phosphine for each species. The susceptible *T. castaneum* population had been maintained in continuous culture for at least 20 years at the USDA-ARS Center for Grain and Animal Health Research (CGAHR), in Manhattan, KS, USA, while the susceptible *R. dominica* population was collected from Arkansas, USA. The phosphine-resistant population of *T. castaneum* was collected from wheat in Palmital, Brazil during 1988 (BRZ-5), while the resistant *R. dominica* population was collected from Oklahoma, USA. The rearing media consisted of 95% organic, unbleached, wheat flour plus 5% brewer's yeast for *T. castaneum* and whole wheat for *R. dominica*. All species were reared at laboratory conditions of 25 °C, and 65% relative humidity (r.h.), in continuous darkness. Adults, of mixed sex and <1 month old, were used in our bioassays.

2.2 Bioassays

The protocol that was used in our bioassays was the standard the Phosphine Tolerance Test (Detia Degesch GmbH, Laudenbach, Germany),[28] with some modifications.[12,19,20] Phosphine was generated and samples were prepared under hooded ventilation. The phosphine was generated within a plastic canister (5 L capacity) by adding 50 mL of water to two kit magnesium phosphide pellets, as described by Steuerwald *et al.*[28] The concentration of the phosphine gas inside the plastic canister was determined by using several Draeger glass tubes (Draeger 25A, 0–10 000 ppm, Draeger Safety AG & Co., USA).[20,28] Ten adults of each population and species were placed in a plastic syringe of 100 mL with separate syringes used for each species and population. Then, a specific gas quantity was removed from the canister with the syringe and blended with fresh air to produce a 100-mL volume with a concentration of either 1000 or 3000 ppm. Ten insects inside the syringe were held for either 15 or 90 min. Additional syringes containing only fresh air and insects were used as negative controls. After each exposure period, the insects were emptied from the syringe and placed into Petri dishes. These insects

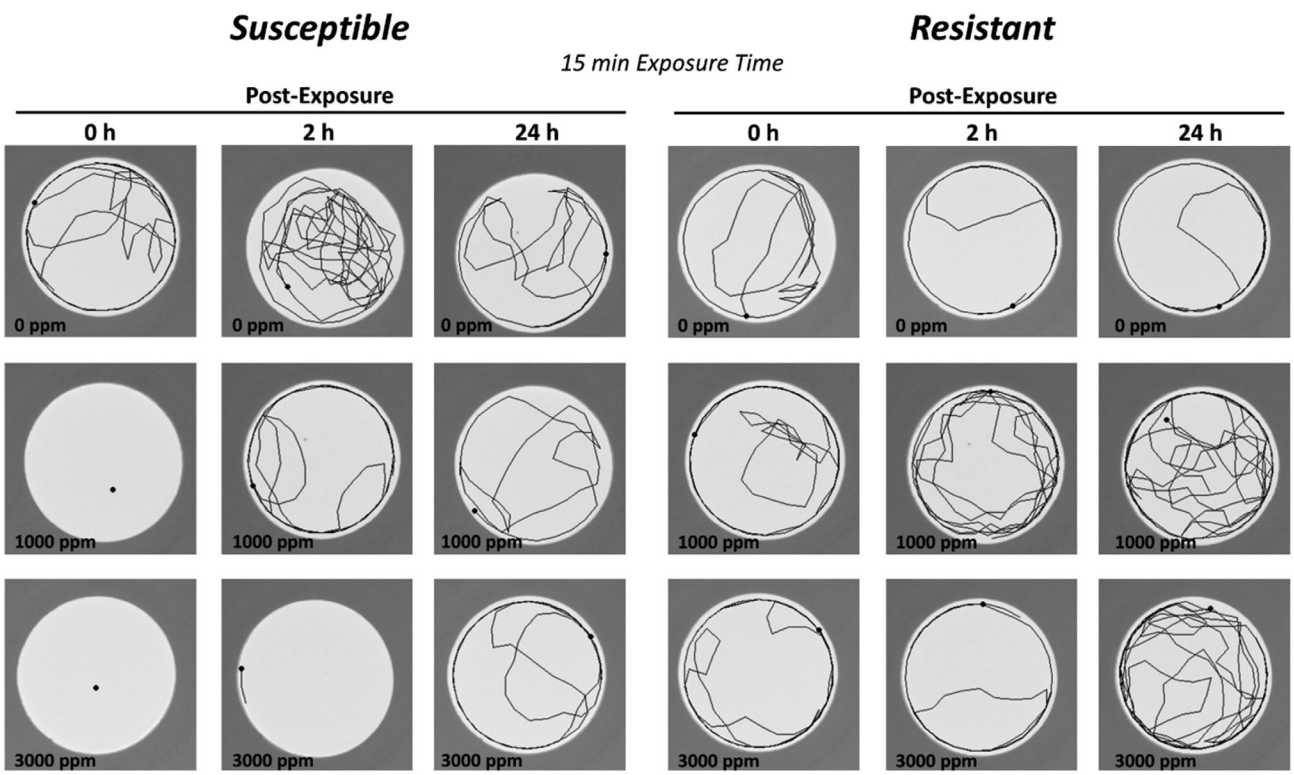

Figure 3. Track visualization of representative movement by phosphine-susceptible (left half) and phosphine-resistant (right half) *Tribolium castaneum* individuals in 100 × 15 mm arenas exposed to 0 (top row), 1000 (middle row) and 3000 ppm (bottom row) for 15 min, with movement recorded immediately after exposure (left most column), 2 h (middle column), or 24 h later (right column). The solid black dot represents the location of the individual at the end of the 15-min sampling period on Ethovision.

were quickly classified visually by the experimenter (under the stereoscope) into two categories: (i) individuals that were knocked down (e.g. narcotized individuals that showed some movement) and (ii) individuals that were dead (immobilized individuals with no visible movement; the classification of these individuals was based on the absence of movement, not to mortality). For the experiments, a total of 144 syringe samples were prepared including syringes for the full factorial combinations of all species (*R. dominica* and *T. castaneum*), populations (susceptible and resistant), concentrations (0, 1000, and 3000 ppm), and exposure times (15 and 90 min), with six replicates per combination.

2.3 Movement pathway measurements

The Ethovision® system XT v. 14.0 (Noldus Software, Leesburg, VA, USA) was described in detail by Guedes *et al.*[22] and Morrison *et al.*[23] From each syringe, three insects were selected randomly and placed individually in each of the three 90 × 15 mm Petri dishes immediately after the post-exposure evaluation. The dish had a piece of filter paper (85 mm D, Grade 1, GE Healthcare, Buckinghamshire, United Kingdom) lining the bottom. Imaging and tracking was conducted on each Petri dish at 0 h (right after the termination of the treatment), 2 and 24 h after exposure to phosphine. Between sample periods, the petri dish of insects were given a pinch of flour 0.2 ± 0.1 g and kept in an incubator set at 25 ± 0.5 °C. The same procedure was followed with control syringes lacking phosphine. The Ethovision® system was setup to monitor six Petri dishes simultaneously. The experiment was synchronized so that two syringe samples were prepared for each 15 min sample period. The network camera (GigE, Basler AG, Ahrenburg, Germany) was mounted 80 cm above the group of six dishes. The movement for a total of 432 insects was recorded, with n = 18 replicate individuals per insect and treatment combination.

2.4 Data analysis

Data were analyzed using SPSS version 25.0 software (SPSS Inc. Chicago, Illinois, USA). Since different concentrations for different exposure intervals were used, the data (knocked down and dead adults) were analyzed following one-way analysis of variance (ANOVA) with concentrations 0, 1000 and 3000 ppm, as main effects. Means of dead and knocked down individuals were separated by Tukey HSD test at $\alpha = 0.05$. For each parameter, to determine differences between susceptible and resistant populations for each species, the data were analyzed using Student's *t*-test. Distance moved (cm) and velocity (cm/min) were the response variables, analyzed separately for each species using one-way ANOVA, as described above.

3 RESULTS

3.1 *Tribolium castaneum*

In the assessments taken immediately after phosphine exposure, the highest percentage of dead adults was achieved at the highest concentration of phosphine (3000 ppm) in susceptible populations of both exposure times, in contrast with the resistant population where immobilization was generally low (Fig. 1). After exposure to phosphine for 15 min, adults of the susceptible populations were 90% dead and approximately 10% were knocked down, whereas all adults were still alive right after exposure to phosphine for the resistant populations (Fig. 1(A),(B)). Significant

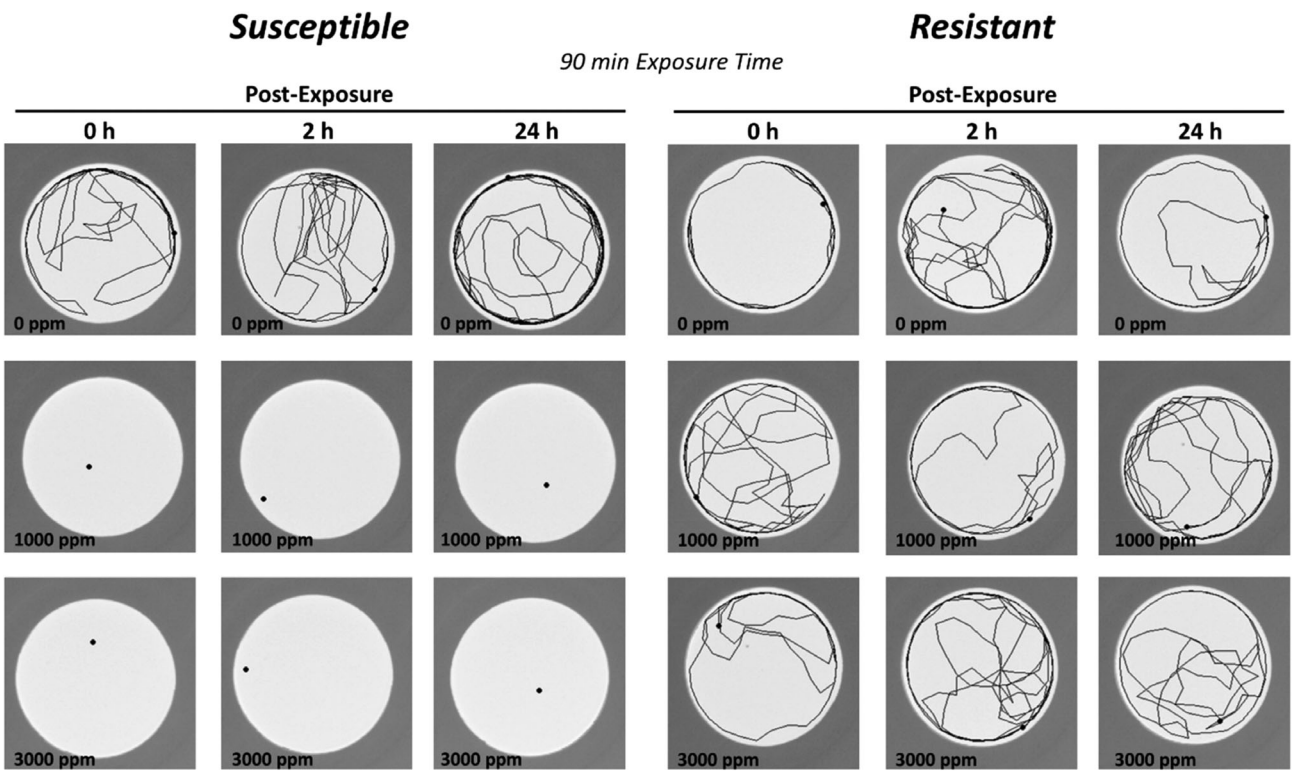

Figure 4. Track visualization of representative movement by phosphine-susceptible (left half) and phosphine-resistant (right half) *Tribolium castaneum* individuals in 100 × 15 mm arenas exposed to 0 (top row), 1000 (middle row) and 3000 ppm (bottom row) for 90 min, with movement recorded immediately after exposure (left most column), 2 h (middle column), or 24 h later (right column). The solid black dot represents the location of the individual at the end of the 15-min sampling period on Ethovision.

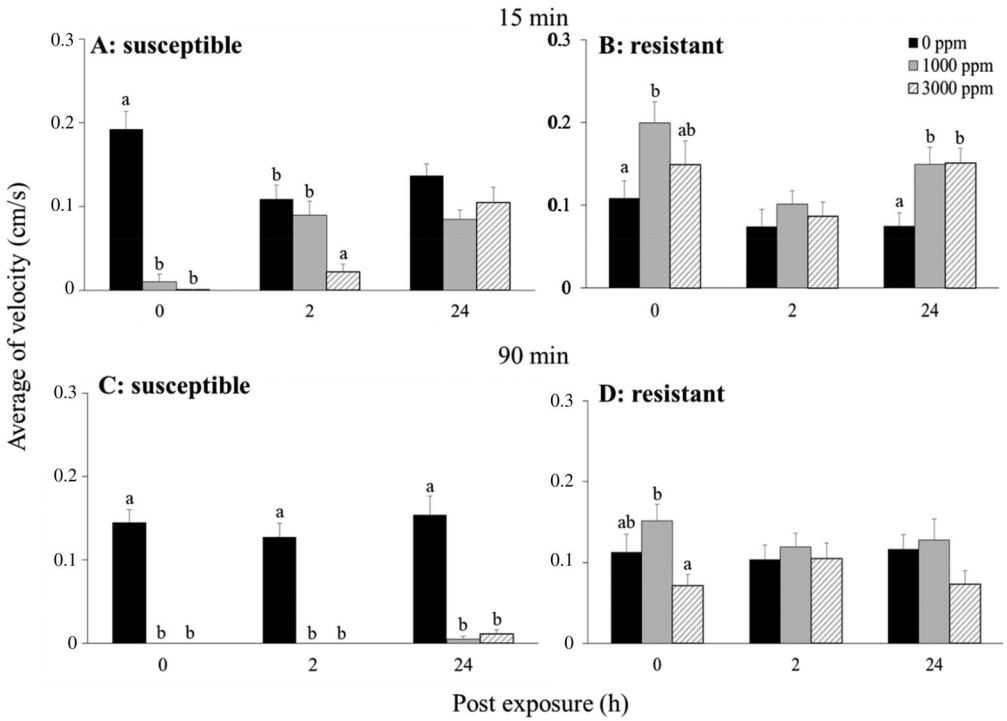

Figure 5. Average of velocity (cm min^{-1}) for *Tribolium castaneum* exposed to 0 (control), 1000 and 3000 ppm for (A) 15 min in susceptible population, (B) 15 min in resistant population, (C) 90 min in susceptible population, (D) 90 min in resistant population. Within each population and post exposure interval, means followed by the same lowercase letter do not differ significantly according to the Tukey–Kramer HSD test at $\alpha < 0.05$. Where no letters exist no significant differences were noted. ANOVA parameters for (A) 0-h F = 62.80, $P < 0.01$, 2-h F = 9.56, $P < 0.01$, 24-h F = 3.20, $P = 0.04$, for (B) 0-h F = 3.20, $P = 0.04$, 2-h F = 0.55 $P = 0.58$, 24-h F = 5.79, $P < 0.01$, for (C) 0-h F = 86.66, $P < 0.01$, 2-h F = 60.40, $P < 0.01$, 24-h F = 38.68, $P < 0.01$ and for (D) 0-h F = 4.30, $P = 0.01$, 2-h F = 0.23, $P = 0.79$, 24-h F = 1.74 $P = 1.85$, in all cases $df = 2, 53$.

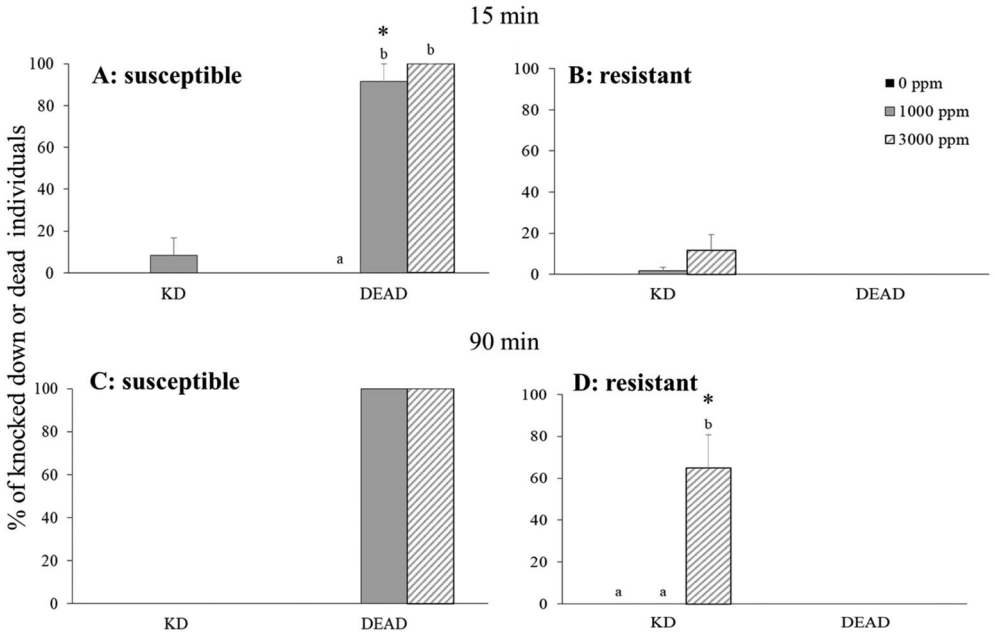

Figure 6. Percentage of knocked down (KD) or dead individuals of *Rhyzopertha dominica* that were exposed to 0 (control), 1000 and 3000 ppm for (A) 15 min in susceptible population, (B) 15 min in resistant population, (C) 90 min in susceptible population, (D) 90 min in resistant population. Within each population and exposure time, means followed by the same lowercase letter do not significantly different according to Tukey–Kramer HSD test at $P < 0.05$. Where no letter exist, no significant differences were noted. ANOVA parameters for knocked down individuals were for (D) F = 16.78, $P < 0.01$, and for dead individuals were for (A) F = 133.0, $P < 0.01$, in all cases $df = 2, 17$. Means with asterisks (*), obtained on susceptible populations, are significantly different from the respective means, obtained on resistant populations, for each exposure interval and concentration (1000 and 3000 ppm), according to Students' *t*-test at $P < 0.05$. According to the *t*-test, the parameters for dead individuals at 1000 ppm were: for (A,B) $t = 11.0$, $P < 0.01$, whereas for knocked down individuals at 3000 ppm were: for (C,D) $t = -4.09$, $P < 0.01$, in all cases $df = 10$. For the rest of the combinations, *t*-test could not be performed.

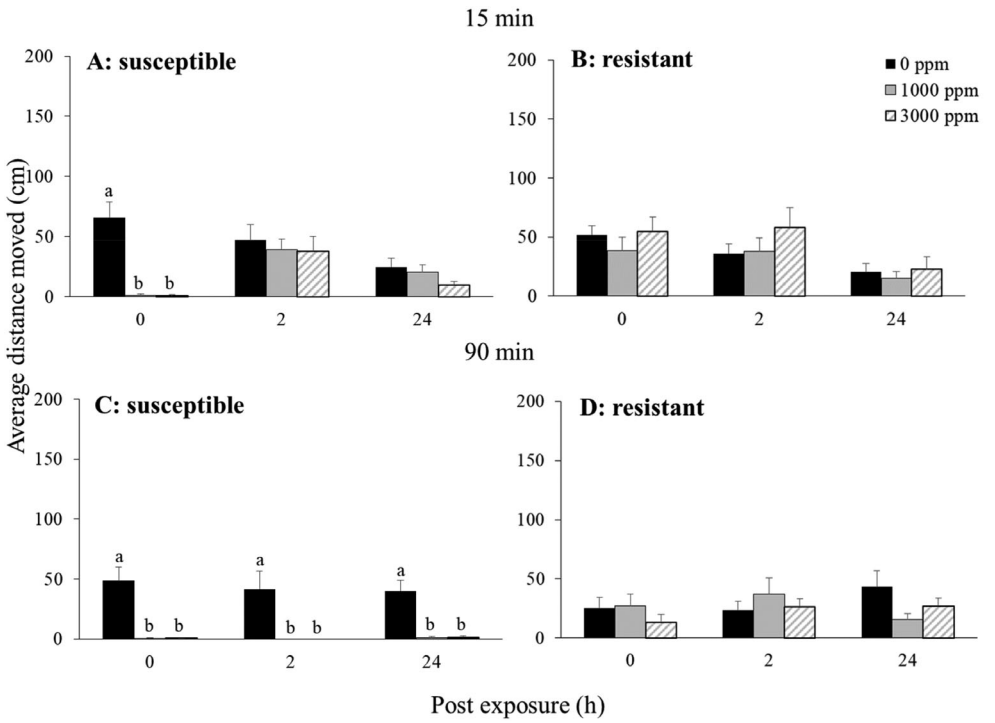

Figure 7. Average distance moved (cm) for *Rhyzopertha dominica* exposed to 0 (control), 1000 and 3000 ppm for (A) 15 min in susceptible population, (B) 15 min in resistant population, (C) 90 min in susceptible population, (D) 90 min in resistant population. Within each population and post exposure interval, means followed by the same lowercase letter do not differ significantly according to the Tukey–Kramer HSD test at $\alpha < 0.05$. Where no letters exist no significant differences were noted. ANOVA parameters for (A) 0-h F = 24.92, $P < 0.01$, 2-h F = 0.18, $P = 0.83$, 24-h F = 1.65, $P = 0.20$, for (B) 0-h F = 0.69, $P = 0.55$, 2-h F = 0.93, $P = 0.40$, 24-h F = 0.19 $P = 0.82$, for (C) 0-h F = 17.23, $P < 0.01$, 2-h F = 7.87, $P < 0.01$, 24-h F = 17.33, $P < 0.01$ and for (D) 0-h F = 0.70, $P = 0.55$, 2-h F = 0.55, $P = 0.57$, 24-h F = 2.29, $P = 0.11$, in all cases $df = 2, 53$.

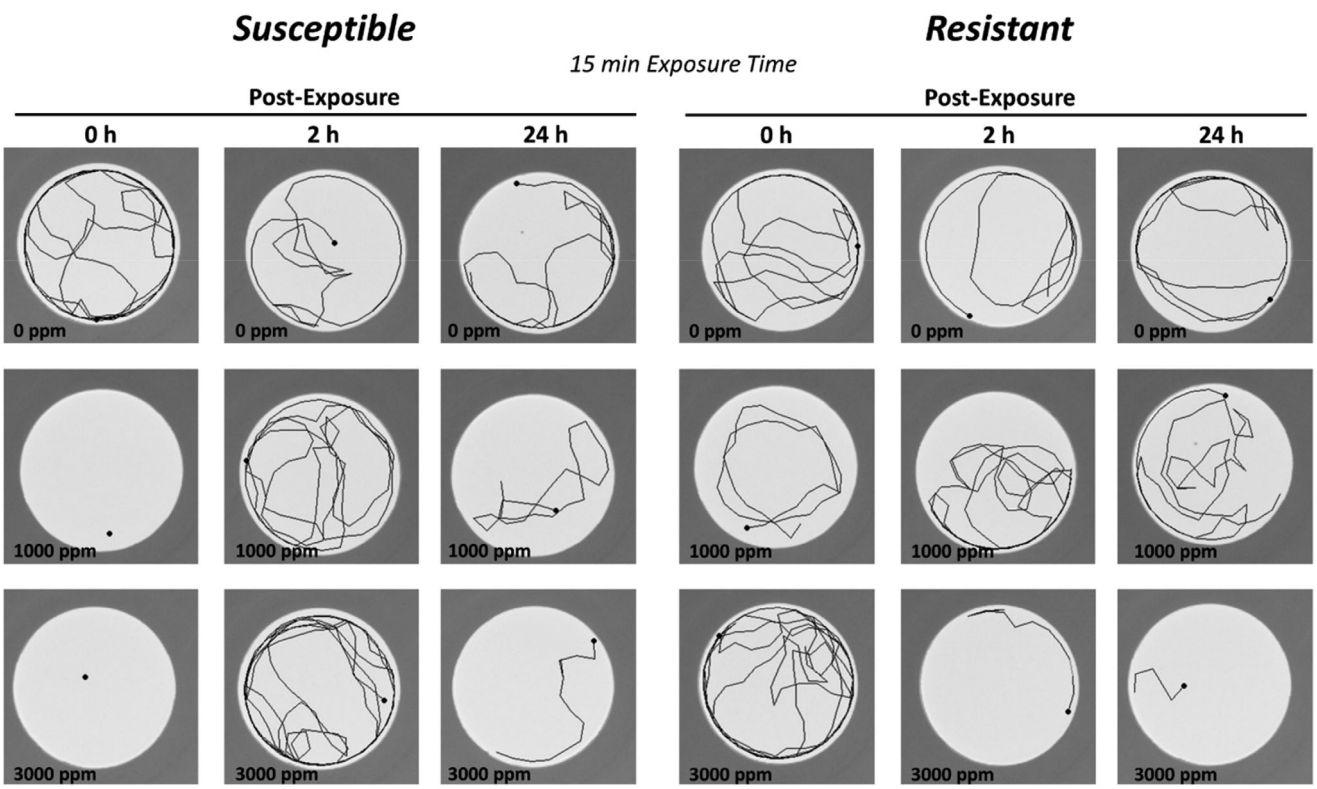

Figure 8. Track visualization of representative movement by phosphine-susceptible (left half) and phosphine-resistant (right half) *Rhyzopertha dominica* individuals in 100 × 15 mm arenas exposed to 0 (top row), 1000 (middle row) and 3000 ppm (bottom row) for 15 min, with movement recorded immediately after exposure (left most column), 2 h (middle column), or 24 h later (right column). The solid black dot represents the location of the individual at the end of the 15-min sampling period on Ethovision.

differences were noted between susceptible and resistant population for each tested concentration (Fig. 1(A),(B)). After exposure to phosphine for 90 min for both concentrations, complete immobilization was noted in the susceptible population (Fig. 1 (C)), while there were only a few dead adults for the resistant population (Fig. 1(D)). For dead individuals, significant differences were observed between the two tested populations (Fig. 1(C),(D)).

The average distance moved ranged from 0 to 180 cm, with considerable variability among treatments and populations (Fig. 2). Immediately after phosphine exposure, the distance movement by susceptible *T. castaneum* was very low at both concentrations, while distance moved increased over time after exposure for the 15 min exposure (Fig. 3) but not at the 90 min exposure (Figs 2(A),(C), 4). By contrast, resistant *T. castaneum* moved just as far after phosphine exposure as in the controls that had only been exposed to air (Fig. 2(B),(D)). At 2-h and onwards, there was likewise similar movement between phosphine-exposed and air-exposed resistant individuals at each concentration (Fig. 2(B),(D)).

Velocity ranged from 0 to 0.2 cm s^{-2} at the 0-h observation, with some variability, shown in Fig. 5. For susceptible individuals, there was no movement and hence no velocity of movement at the 0-h observation, with the exception of the control (0 ppm), but the velocity rebounded at 2- and 24-h after 15-min exposures to phosphine at each concentration, though not for the longer 90-min exposure times. By contrast, the velocity of resistant *T. castaneum* was largely not impaired by exposure to phosphine compared with the controls, even when exposed at higher concentrations for longer periods of time.

3.2 *Rhyzopertha dominica*

A high percentage of dead adults were detected at both concentrations and exposure times for the susceptible population (Fig. 6 (A),(C)). For dead individuals at 15 min, significant differences were noted among the tested concentrations (0, 1000 and 3000 ppm) (Fig. 6(A)), while at 90 min all individuals were dead (Fig. 6(C)). In contrast, for the resistant population there was little impact of exposure except for higher levels of knockdown at the 3000 ppm and 90 min exposure period treatment (Fig. 6(B),(D)).

The average distance moved ranged from 0 to 60 cm, with large variability among the treatments and strains (Fig. 7). Immediately after exposure for 15 min, there were no movements at susceptible population of *R. dominica* in the controls however, distance moved by susceptible *R. dominica* exposed to 1000 and 3000 ppm of phosphine quickly recovered 2- and 24-h after exposure (Figs 7(A),(C), 8). Significant differences were noted within each exposure interval (0, 2 and 24 h) for 90 min among the tested concentrations (Fig. 7(C)). By contrast, during longer 90 min exposure to phosphine the distance moved by susceptible *R. dominica* was only appreciable in the air-exposed controls even 2- and 24-h after exposure (Fig. 9). By contrast, none of the resistant *R. dominica* exposed for either duration at either concentration experienced reduced movement compared to controls (Fig. 7(B),(D)).

Velocity ranged from 0 to 0.08 cm s^{-1} at the 0-h observation (Fig. 10). As above, immediately after exposure to phosphine for 15 min, susceptible *R. dominica* moved very little compared with controls while the distance moved quickly rebounded at 2- and 24-h post-exposure at 1000 and 3000 ppm phosphine

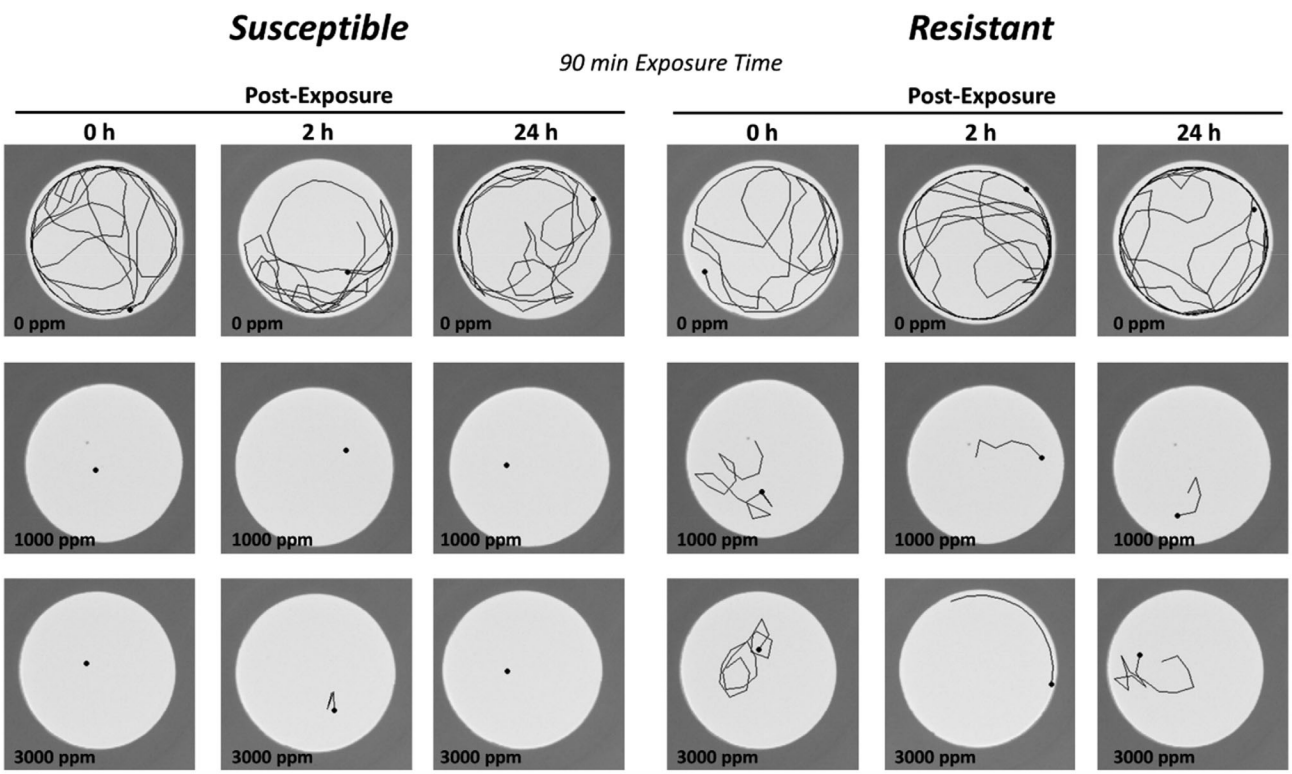

Figure 9. Track visualization of representative movement by phosphine-susceptible (left half) and phosphine-resistant (right half) *Rhyzopertha dominica* individuals in 100 × 15 mm arenas exposed to 0 (top row), 1000 (middle row) and 3000 ppm (bottom row) for 90 min, with movement recorded immediately after exposure (left most column), 2 h (middle column), or 24 h later (right column). The solid black dot represents the location of the individual at the end of the 15-min sampling period on Ethovision.

concentrations (Fig. 10(A),(C)). Figure 10(C) showed that there were significant differences of insect movement velocity among the tested concentrations within each exposure interval. Susceptible *R. dominica* exposed to phosphine for 90 min never recovered much movement compared to controls. In contrast, resistant *R. dominica* individuals did not show an appreciable reduction in velocity at either concentration or exposure time (Fig. 10(B),(D)).

4 DISCUSSION

The use of different terms to describe behavioral changes after exposure to phosphine has often been a source of confusion rather than an objective diagnostic factor of fumigant efficacy. The same holds true in the case of the contact insecticides, as the classification of an insect as 'knocked down' may vary according to the individual that is taking the measurements. For instance, Baliota *et al.*[29] found that knocked-down beetles after exposure to contact insecticides exhibited different mobility characteristics, ranging from vigorous, but still abnormal, movement, to minimal movement of the antennae and the tarsi. Traditionally, to describe behavioral changes during exposure to phosphine, many authors have used the term 'knockdown' which illustrates deviations from walking normally to minimal body movements.[7,9,11,12,16,30] Nayak *et al.*[9] used knockdown as an indicator of susceptibility, weak or strong resistance for populations of the rusty grain beetle, *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae). An older series of publications used the term 'narcosis' to describe the changes in behavior tending towards complete immobilization.[31,32] The somewhat arbitrary definition of key terms associated with insecticides by different authors has also been demonstrated for 'broad spectrum'.[33] In addition to this linguistic uncertainty, immobilization during exposure may not be necessarily indicate increased susceptibility, as recovery may often ensue after some period of time.[18,19] In the current work, we observed that each species and population responded in a different way, but typically with reduced mobility right after exposure to phosphine, even at the 15-min exposure interval, indicting the speed of intoxication by this gas.

Not surprisingly, the measurements that were taken visually (under the stereoscope) were considerably different than those that were recorded by Ethovision®. The visual observations separated the adults as 'moving' or 'not moving', providing a 'pass-fail' estimate of the exposed individuals. This pass-fail evaluation has been used in many diagnostic methods, especially the ones that provide immediate estimations, and which do not include an evaluation of recovery.[9,20,28] However, often, it is recovery after exposure that indicates the presence of resistance and not immobilization during the exposure.[8,10,12,19] For the same phosphine resistant *T. castaneum* population used here, Athanassiou *et al.*[19] observed that despite the immediate response, post-exposure recovery rates were high, even 2-h after the exposure.

Our current findings show that between concentrations of 1000 and 3000 ppm of phosphine, the number of dead adults increased at the higher concentration for the susceptible populations of each species tested. Paradoxically at 3000 ppm, increasing the exposure from 15 to 90 min did not increase the number of knocked down resistant *T. castaneum* adults. Moreover, this was not combined with a proportional increase in the

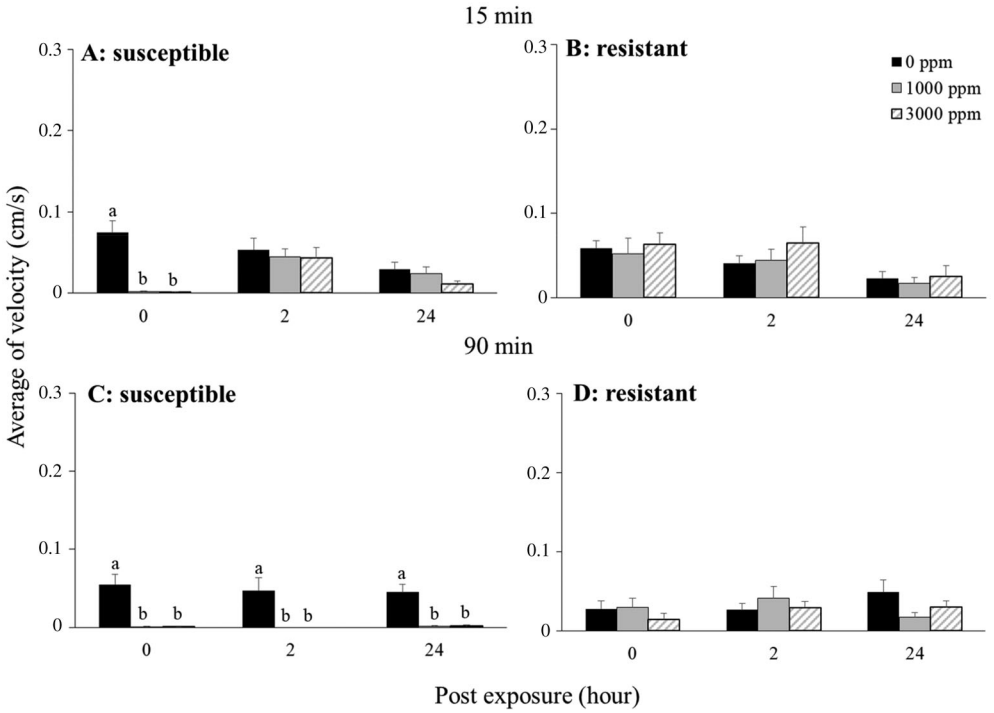

Figure 10. Average of velocity (cm min^{-1}) for *Rhyzopertha dominica* exposed to 0 (control), 1000 and 3000 ppm for (A) 15 min in susceptible population, (B) 15 min in resistant population, (C) 90 min in susceptible population, (D) 90 min in resistant population. Within each population and post exposure interval, means followed by the same lowercase letter do not differ significantly according to the Tukey–Kramer HSD test at $\alpha < 0.05$. Where no letters exist no significant differences were noted. ANOVA parameters for (A) 0-h F = 25.39, $P < 0.01$, 2-h F = 0.20, $P = 0.82$, 24-h F = 1.17, $P = 0.19$, for (B) 0-h F = 25.39, $P = 0.84$, 2-h F = 0.84, $P = 0.43$, 24-h F = 0.19, $P = 0.82$, for (C) 0-h F = 17.72, $P < 0.01$, 2-h F = 8.11, $P < 0.01$, 24-h F = 18.16, $P < 0.01$ and for (D) 0-h F = 0.70, $P = 0.50$, 2-h F = 0.51, $P = 0.60$, 24-h F = 2.43, $P = 0.09$ in all cases $df = 2, 53$.

percentage of the dead adults for the resistant population. This clearly suggests that the response of the adults by this resistant population, and probably other resistant populations, to an increase in the concentration of phosphine is not linear and to some extent may be reversed.[19] Reduction in immobilization at elevated concentrations of phosphine was initially described by Winks[31] and Winks and Waterford.[32] For *T. castaneum*, the authors found that there was unexplained recovery at elevated concentrations, and postulated that this phenomenon might have been caused to alterations in interactions of the exposed insects with phosphine.[19,20] From a practical point of view, this elevated response should be examined in more detail, as it may be related with reduced mortality at food facilities.

As noted above, there were considerable deviations between visual observations and actual movement parameters, as these were estimated by Ethovision®. Apparently, there was an immediate delay in movement by the susceptible populations, for both species, when exposed to either 1000 and 3000 ppm. For the susceptible populations of *T. castaneum* and *R. dominica*, the effect of phosphine on insect movement was partially ameliorated at 2 and 24 h after exposure when exposed for 15 min, but the effects of phosphine on movement were irreversible after 90-min exposures, which supports prior work.[19] Conversely, resistant *T. castaneum* adults were significantly more mobile than the susceptible adults immediately after exposure to 1000 ppm for 15 or 90 min. Thus, short exposures to phosphine may be an irritant or trigger an escape response in resistant *T. castaneum* population. The same trend was also expressed in the case of the resistant *R. dominica* population, though to a lesser extent, demonstrating that this may be a general response by stored product insect species that are resistant to phosphine. Interestingly, 2 and 24 h after exposure to phosphine, resistant insects significantly recovered and appeared just as mobile as controls, suggesting that their dispersal may be further aided by adaptations in their biology to tolerate phosphine, which may allow insects to persist after a fumigation if refugia are sought out by individuals.

We found that the susceptible populations exposed to air for each species moved longer distances and at higher velocity levels compared with the resistant populations exposed to air. There are several studies that provide dissimilar and often contradictory conclusions about mobility in relation to resistance, using indicators that range from previous exposure to insect 'personality'.[27,34–38] Pimentel *et al.*[35] showed that reduced mobility was related with lower respiration rates in *R. dominica*, resulting in reduced phosphine uptake, which itself may be connected with energy production and flow, a critical parameter in phosphine's mode of action. By contrast, Kaur *et al.*[34] reported that there were no differences in walking and flight behavior between phosphine-susceptible and -resistant *R. dominica* populations. Similarly, in a recent study, Sakka *et al.*[27] found that the vast majority of walking parameters of populations for *T. castaneum* and *R. dominica* with differential susceptibility levels to phosphine did not differ significantly. However, our study shows that there are differences between susceptible and resistant strains in mobility, suggesting that basic respiration patterns should be further investigated, as it is related with phosphine uptake.[36] In this context, automated video-tracking tools such as Ethovision® can serve as an useful quantification tool, which can then be associated with corresponding physiological characteristics (carbon dioxide production, energy flow *etc.*) of individuals.

Despite Ethovision® being primarily a laboratory research tool, which is not accessible by the wide audience due to cost restrictions, some generalizations can be drawn towards beetle behavior after exposure to phosphine.

Our study used visual observations as well as detailed, quantified mobility parameters to characterize recovery of phosphine-exposed individuals, and, to some extent, the 'speed to recovery', as a measurement of resistance. We clearly observed behaviorally plastic responses in resistant populations after exposure to phosphine, especially in the case of *T. castaneum*, which persisted for longer than expected. We also saw interesting responses to changes in both concentration and exposure intervals, which may correspond to non-linear changes in phosphine uptake. Additional insect response data should be collected and examined at greater depth including both short term and long term exposure periods. Then, the effectiveness of the length of exposure could be assessed for potential industry fumigation practices.

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