Journal of Stored Products Research 90 (2021) 101737

Contents lists available at ScienceDirect

Journal of Stored Products Research

journal homepage: www.elsevier.com/locate/jspr

Immediate and delayed effects of short exposures to phosphine on adults and larvae of the khapra beetle, *Trogoderma granarium*

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ARTICLE INFO

Article history:
Received 18 June 2020
Received in revised form
21 August 2020
Accepted 31 October 2020
Available online xxx

Keywords:
Quarantine pest
Khapra beetle
Short exposures
Diagnostic test
Immobilization
Phosphine
Tolerance

ABSTRACT

Adults and larvae of the khapra beetle, *Trogoderma granarium* Everts were exposed to 3000 ppm of phosphine through the Phosphine Tolerance Test. In a first series of bioassays, observations were taken every 2 min and the exposed individuals were classified either as walking normally or as being immobilized (knocked down), i.e., not walking normally. In the second series of bioassays all individuals were exposed for 90 min to phosphine. For both bioassays delayed mortality was noted after a 7 and 14-day post exposure interval. Larvae were found to be more tolerant than adults, as the time required for the individuals to be immobilized was up to 20 min, which was almost twice as long as the time required for the immobilization of the adults. There were high levels of adult mortality 7 days later, and complete (100%) mortality 14 days after the exposure. In contrast, larval mortality was low, for both post-exposures. Adults were 100% immobilized after the termination of the 90-min exposure interval at 3000 ppm of phosphine, while the percentage of the active larvae was extremely low. Regarding the 7 days post exposure interval the percentage of larval immobilization was higher than that of adults, but this was reversed seven days later. Interestingly, development of the larvae was delayed compared to the control, after the 90 min exposure, in contrast to 20 min exposure which did not cause any delayed effect. Our work provides some first data for the evaluation of the influence of short exposures to phosphine on adults and larvae of *T. granarium,* which may be very useful in creating an effective initial quantification plan for the control of this species.

1. Introduction

The khapra beetle, *Trogoderma granarium* Everts (Coleoptera: Dermestidae) is an important pest of stored products and has been listed as one of the 100 worst invasive species worldwide (Myers and Hagstrum, 2012; EPPO, 2013) and a quarantine species in many countries (Athanassiou et al., 2019a). This species is able to cause extremely high infestation levels to a wide range of stored products (Hagstrum and Subramanyam, 2009; Athanassiou et al., 2019a; Kavallieratos et al., 2019). The life stage that causes the infestation is the larvae, while adults do not feed (Pasek, 1998; Athanassiou et al., 2019a). Moreover, the larvae of *T. granarium* are tolerant to many chemical and non-chemical control methods, especially when then are in diapause (Bell and Wilson, 1995; Ghimire et al., 2016, 2017; Kavallieratos et al., 2017; Athanassiou et al., 2019a). Indicatively, Kavallieratos et al. (2017) found that large larvae of this species were less susceptible than small ones to contact insecticides, while Wilches Correal (2016) reported that diapausing and acclimated *T. granarium* larvae were tolerant to low and high temperatures that are usually lethal for other major stored product insects.

Phosphine, or hydrogen phosphide, is currently one of the most important insecticides in stored product protection, while its importance for post-harvest phytosanitary applications has been increased after the withdrawal of methyl bromide (UNEP, 1997). Considering the phytosanitary importance of *T. granarium*, further research on phosphine towards the control of this species should be considered a priority (Nayak et al., 2020). Currently, although many studies demonstrate the efficacy of phosphine to major stored product insects, the data related with *T. granarium* are disproportionally few. In the Journal of Stored Products Research, during the last decade, there were eight articles regarding the efficacy of

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https://doi.org/10.1016/j.jspr.2020.101737
0022-474X/

phosphine on the lesser grain borer, *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae) and ten on the red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) while there was only one article for *T. granarium* and phosphine, and this very recent (Yadav et al., 2020). This is partially due to the fact that many laboratories throughout the globe do not have *T. granarium* colonies, since it requires compliance with certain phytosanitary regulations and protective measures (Athanassiou et al., 2019a).

As in the case of other stored product insect species, the occurrence of resistant populations of *T. granarium* has been reported in some parts of the world (Benhalima et al., 2004; Ahmendani et al., 2007; Athanassiou et al., 2019b, 2019c). The evaluation of resistance is based upon different types of methods, with some of them being less laborious than others (Nayak et al., 2020). From these methods, the "rapid diagnostic" methods mostly rely on insect immobilization after exposure, usually mentioned as "knockdown". Theoretically, the "speed to knockdown" may be related with the "speed of kill", in terms of mortality during or after the exposure. Sakka et al. (2018) found that this relationship worked well in most of the populations tested, and that the populations that had the highest knockdown times were the ones that were the most susceptible to phosphine. Based on this, Athanassiou et al. (2019c) provided times-to-immobilization that can be used with success for the quantification of susceptibility to phosphine of several major stored-product beetle species. These times are given in minutes, and estimated through exposure at 3000 ppm of phosphine using the Phosphine Tolerance Test (PTT, Detia Degesch GmbH, Germany). The use of these indicators are expected to provide a very quick and accurate detection and estimation, that can be utilized further for planning "real world" fumigation practices, based on specific characterizations of the insect populations that are to be controlled (Athanassiou et al., 2019b, 2019c). Nevertheless, there are published data that show that the results from a quick diagnostic may not be correlated accurately to more time-consuming resistance evaluation methods. For instance, Agrafioti et al. (2019) found that the results from PTT correlated well with the 20-h exposure protocol of the Food and Agriculture Organization (FAO) for all species tested, with the exception of the granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae). However, this quick diagnostic has not been tested for *T. granarium*.

The aim of the present study was to use for the first time this quick diagnostic test, in the case of *T. granarium*, to identify a "time to knockdown" for this species as well, along with the delayed effects of the exposure. Taking into account that adults of this species are short-lived, in contrast with adults in the vast majority of stored product beetle species, and considering the tolerance of *T. granarium* larvae to various insecticides, we performed the PTT in parallel for both life stages.

2. Materials and methods

2.1. Insects

The individuals used in this series of bioassays were adults and larvae from a colony maintained at the Laboratory of Entomology and Agricultural Zoology (LEAZ), Department of Agriculture, Crop Production and Rural Environment, University of Thessaly since 2013 (Athanassiou et al., 2016). The rearing conditions were 32 °C and 55% relative humidity (r.h.), in continuous darkness. Both adults and larvae had been separated from the colonies using a 2 mm sieve (Woven Wire Sieve, Endecotts Ldt, London, England). Subsequently we used a 850 μm sieve (Woven Wire Sieve, Endecotts Ldt, London, England) in order to separate the larger larvae from the smaller ones. Both larvae and adults were collected with a fine paint brush (Lineo, No.1, Mesko-Pinsel GmbH). Adults of mixed sex, and approx. 2 d-old, were used in the bioassays.

2.2. Twenty minute bioassays

We used the PTT as described by Agrafioti et al. (2019) and Athanassiou et al. (2019b, 2019c). Briefly, we used the PTT canister to produce phosphine, using the standard test tablets. The determination of the concentration of phosphine inside the canister was held by using glass tubes (Draeger 25A, Draeger Safety AG & Co., Germany) (Steuerwald et al., 2006; Athanassiou et al., 2019b, 2019c). Ten individuals of either adults or larvae were introduced into the 100 ml syringe of the test that contained 3000 ppm of phosphine. To pursue this concentration in the total air volume of 100 ml, a specific gas quantity was removed from the canister according to the manual of the test while the rest volume was filled with air. Additional syringes with insects, containing only air, were used as controls. The individuals were classified as immobilized, i.e. individuals that could not move normally as compared to the controls and active, i.e. individuals that were capable for coordinated movement, as in the case of the controls. The mobility of the individuals was recorded visually every 2 min, until the time that all exposed individuals were immobilized. After the termination of this interval, the insects were removed from the syringes and were transferred to petri dishes in the open air with a small amount of food, where delayed mortality was observed 7 and 14 d later. In the case of the exposed larvae, we also recorded their eventual life stage for both post exposure intervals. There were three replicates with three sub-replicates (9 syringes in total), with new phosphine production for each replicate.

2.3. Ninety minute bioassays

In this bioassay adults and larvae of the tested population were exposed to 3000 ppm of phosphine for 90 min, using the same procedure as above. The insects were exposed for 90 min, and then transferred to petri dishes, on which immediate response were observed, by classifying them either as active or as immobilized. Delayed effects' observations, as well as the number of replicates and subreplicates, were as above.

2.4. Data analysis

For the 20 min bioassays data were analyzed by using Probit Analysis to estimate the lethal time, i.e., LT_{50}, LT_{95} and LT_{99} for both life stages tested. For the delayed effect, all data were submitted to *t*-test in order to compare larval to adult mortality for both post exposure intervals (7 and 14 days post-exposure time). In addition, we performed a *t*-test to compare the development of exposed larvae which survived the exposure, with the respective figures in the controls. Regarding the 90 min bioassays, all data were submitted to *t*-test separately for each interval to compare larval and adult mortality. Moreover, there was also a developmental comparison of the delayed effect of the 90 min bioassays, as above. In all cases we used SPSS version 25 (IBM Corp., 2016). There was no adult and larval control mortality during the exposure period, for both exposure intervals (20 and 90 min).

3. Results

3.1. Twenty minute bioassays

Larvae were found to be more tolerant than adults, as the time required for the larvae to be immobilized was up to 20 min, which was almost twice as long as the time required for the

immobilization of the adults (Fig. 1). Probit analysis fit the data adequately for both life stages, with LT_{99}, determined as 8.8 and 15.9 min for adults and larvae, respectively (Table 1). At the 7-d post exposure period, adult mortality was approx. 85%, while all adults were dead 7 days later (Table 2). In contrast, larval mortality was low, for both post exposures. There was a low adult survival (approx. 15%), 7 days after the exposure which led to complete mortality after the 14-d post exposure interval. In contrast, we recorded high survival levels of the exposed larvae, 7 days after the exposure, which was slightly decreased 7 days later (approx. 90 and 75%, respectively) (Table 2). Regarding the delayed effects of phosphine on the exposed larvae, we found that there were no significant differences as compared to the control larvae (Table 3). In general, 7 and 14 days after the termination of the exposure, most of the larvae became pupae and adults, respectively.

3.2. Ninety minute bioassays

Adults were 100% immobilized after the termination of the 90-min exposure interval at 3000 ppm of phosphine, while the percentage of the active larvae was extremely low (Table 4). Interestingly, for this series of bioassays, at the 7-d post exposure period, the percentage of larval immobilization was higher than that of adults, but this was reversed seven days later. Moreover, at the 7-d post exposure period, approx. one third of the exposed larvae were still at the larval stage, while all larvae in the controls were recorded as either pupae or adults (Table 5). Similarly, at the 14-d post exposure period, 22% of the exposed larvae were still at the larval stage, while almost all control larvae had been emerged as adults. In contrast, only approx. one third of the exposed larvae were emerged as adults at this post exposure period (Table 5).

Table 2
Mortality (% ± SE) of adults and larvae of *T. granarium* 7 or 14 days after the termination of the exposure to 3000 ppm of phosphine (in all cases total $df = 16$).

	Post exposure intervals	
Life stage	7 d	14 d
Adults	85.5 ± 5.3*	100.0 ± 0.0*
Larvae	5.5 ± 3.4	26.7 ± 4.1
t	12.72	17.96
P	<0.001	<0.001

*Within each column, an asterisk indicates significant differences; Students' *t*-test at 0.05.

4. Discussion

Our results for PTT show that the time to immobilization of *T. granarium* adults was comparable with this that has been reported for most stored product beetle species by Athanassiou et al. (2019c). However, in that study, the authors examined several laboratory populations to estimate time to immobilization for other species (Athanassiou et al., 2019c), so using one single population cannot be used alone to draw generalized conclusions. We also found that immobilization of larvae takes more time than adults. For many stored product species, larvae are considered more tolerant than adults to phosphine (Howe, 1973; Hole et al., 1976; Kaur et al., 2012). For instance Vincent and Lindgren (1972) exanimated the susceptibility of different life stages of four Dermestidae, the black carpet beetle, *Attagenus megatoma,* (F.), the glabrous cabinet beetle, *Trogoderma glabrum* (Herbst), *T. sternale* Jayle and the warehouse beetle, *T. variabile* Ballion and verified stronger tolerance of the larval stage. Nevertheless, for the larger cabinet beetle, *Trogoderma inclusum* LeConte (Coleoptera: Dermestidae), Athanassiou et al. (2020) found that adults and larvae were equally susceptible to phosphine; still the exposure intervals

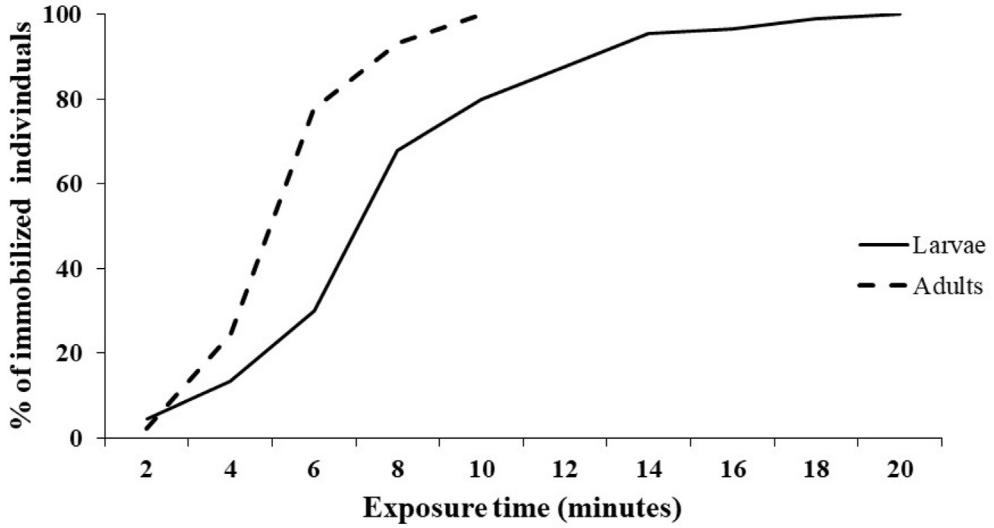

Fig. 1. Percentage (%) of immobilized *T. granarium* adults (dashed line) and larvae (solid line), after exposure to phosphine at 3000 ppm for different observation intervals (in min.).

Table 1
Probit analysis for LT_{50}, LT_{95}, LT_{99} (confidence internals) of adults and larvae of *T. granarium* after exposure to 3000 ppm of phosphine using the PTT.

Life stage	*df*	LT_{50}	LT_{95}	LT_{99}	Slope	x^2	*P*
Adults	3	5.0 (3.3–6.0)	7.7 (6.6–10.7)	8.8 (7.4–13.2)	3.4 ± 0.1	0.5	0.918
Larvae	8	7.5 (5.1–9.2)	13.5 (11.6–16.6)	15.9 (13.6–20.3)	5.0 ± 0.0	2.2	0.973

Table 3
Percentage (±SE) of larvae, pupae and adults of *T. granarium*, found 7 and 14 d after the termination of the 20 min larval exposure to 3000 ppm of phosphine, and the respective figures in the control larvae (in all cases, within each line, larvae + pupae + adults = 100%; total *df* = 16).

	Post exposure interval					
	7 d			14 d		
	larvae	pupae	adults	larvae	pupae	adults
Exposed larvae	1.1 ± 1.1	81.1 ± 6.5	17.7 ± 6.6	0.0 ± 0.0	9.1 ± 5.2	90.8 ± 5.2
Control	0.0 ± 0.0	80.0 ± 8.8	20.0 ± 8.8	0.0 ± 0.0	7.5 ± 3.4	92.4 ± 3.4
t	1.00	0.10	-0.20	–	0.26	-0.26
P	0.332	0.919	0.842	–	0.797	0.797

*Within each column, an asterisk indicates significant differences; Students' *t*-test at 0.05.

Table 4
Percentage (±SE) of adults and larvae of *T. granarium* that were found immobilized after 90 min of exposure to 3000 ppm of phosphine and after a 7 and 14-day post exposure period (in all cases total *df* = 16).

	Exposure/Post exposure intervals		
Life stages	90 min	7 d	14 d
Adult	100.0 ± 0.0	30.0 ± 8.8*	97.8 ± 1.5*
Larvae	98.9 ± 1.1	72.2 ± 7.2	61.1 ± 6.5
t	1.0	-3.7	5.4
P	0.332	0.002	<0.001

*Within each column, an asterisk indicates significant differences; Students' *t*-test at 0.05.

tested in that study were far higher than the ones tested here.

Not surprisingly, the post exposure mortality of the adults was high, and eventually reached 100%, suggesting that adults were affected even by the short exposures used in the current bioassays. It should be noted though that adults of this species are short-lived (Athanassiou et al., 2019a) and hence, high mortality is expected even without previous exposure to phosphine. Nevertheless, for populations of other stored product beetle species that are susceptible to phosphine, even an exposure as short as 15–90 min to 3000 ppm can be lethal to adults (Sakka et al., 2018; Athanassiou et al., 2019b, 2019c; Agrafioti et al., 2019). Conversely, recovery of larvae was high, despite the fact that larval mortality was increased with the increase of the post exposure period. Moreover, for the 20-min exposure, larvae continued their growth and metamorphosis at similar levels with the control larvae. The post exposure conditions used in our study were considered suitable for the larvae to complete their development and eventually emerge as adults. It is generally accepted that temperatures that are lower than 30 °C, in conjunction with larval overcrowding, may force larvae to induce diapause, rather than to continue their development at the pupal stage (Burges, 1962; Nair and Desai, 1973; Bell, 1994; Wilches et al., 2016; Shivananjappa et al., 2020). Wilches et al. (2016) considered as daupausing larvae, recently hatched larvae which had not pupated after 45 days at 30° C.

Interestingly, when larvae were exposed for 90 min to phosphine, their development was notably affected, as compared with the control larvae. Despite the fact that a considerable percentage of larvae survived the 90-min exposure, most of these larvae exhibited a notable delay in their development, and remained at the larval stage much longer than the control larvae. In fact, for the treated larvae, adult emergence was low even at the 14-d post exposure interval. After exposing psocid eggs of *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae) of a resistant and a susceptible population to phosphine, Nayak et al. (2003) observed delayed hatching, for both populations, and especially for the resistant one. Still, we are unaware about the eventual survival, longevity and fecundity of the exposed individuals, since we examined only a 14-d interval. This delay in development may be related with specific fitness mechanisms that allows developmental adaptations when the conditions prevailing are not suitable. Furthermore, a temporary delay may result in increased survival after the application of phosphine, and increased risks from the presence of dormant individuals in the treated areas.

Our work provides some first data for the evaluation of the influence of short exposures to phosphine on adults and larvae of *T. granarium*. Considering the utilization of PTT, we found times that are similar to those that had been reported in the case of other species. Moreover, when the exposure was increased to 90 min, we have found an interesting developmental inhibitory effect of phosphine to the exposed larvae, that merits additional investigation, on the basis of the hypothesis of a possible biological adaptation during or after sub-lethal exposures.

CRediT authorship contribution statement

Marina Gourgouta: Conceptualization, Methodology, Formal analysis, Writing - original draft, Investigation. **Paraskevi Agrafioti:** Conceptualization, Methodology, Formal analysis, Investigation, Resources, Project administration, Writing - original draft. **Christos G. Athanassiou:** Writing - review & editing, Supervision, Funding

Table 5
Percentage (±SE) of larvae, pupae and adults of *T. granarium*, found 7 and 14 d after the termination of the 90 min larval exposure to 3000 ppm of phosphine, and the respective figures in the control larvae (in all cases, within each line, larvae + pupae + adults = 100%; total *df* = 16).

	Post exposure interval					
	7 d			14 d		
	larvae	pupae	adults	larvae	pupae	adults
Exposed larvae	29.9 ± 6.5*	70.0 ± 6.5	0.0 ± 0.0*	22.2 ± 8.5*	41.8 ± 12.2*	35.9 ± 6.3*
Control	0.0 ± 0.0	85.0 ± 4.7	14.0 ± 4.7	0.0 ± 0.0	4.9 ± 2.7	95.0 ± 2.7
t	4.57	-1.85	-3.14	2.58	2.92	-8.58
P	<0.001	0.083	0.006	0.020	0.010	<0.001

*Within each column, an asterisk indicates significant differences; Students' *t*-test at 0.05.

acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the project NANOFUM T2DGE-0917 (co-funded by the European Union and Greek National Funds through the Operational Program Competitiveness, Entrepreneurship and Innovation – EPAnEK 2014-2020, NSRF 2014-2020, Ministry of Development & Investments / Special Secretary for Management of ERDF and CF Sectoral Operational Programmes). Action: Bilateral R & T cooperation between Greece and Germany. This paper reports the results of research only. Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by CERTH/IBO.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jspr.2020.101737.

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