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Phosphine Resistance in Adult and Immature Life Stages of *Tribolium castaneum* (Coleoptera: Tenebrionidae) and *Plodia interpunctella* (Lepidoptera: Pyralidae) Populations in California

S. G. Gautam,[1,2] G. P. Opit,[1,3] and E. Hosoda[4]

[1]Department of Entomology and Plant Pathology, Oklahoma State University, 127 Noble Research Center, Stillwater, OK 74078 (sangautam@ucanr.edu; george.opit@okstate.edu), [2]Current address: Kearney Agricultural Research and Extension Center, 9240 S Riverbend Ave., Parlier, CA 93648, [3]Corresponding author, e-mail: george.opit@okstate.edu, and [4]Cardinal Professional Products, 57 Matmor Rd., Woodland, CA 95776 (ehosoda@cardinalproproducts.com)

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Abstract

Phosphine resistance in stored-product insects occurs worldwide and is a major challenge to continued effective use of this fumigant. We determined resistance frequencies and levels of resistance in *Tribolium castaneum* and *Plodia interpunctella* collected from California almond storage and processing facilities. Discriminating doses of phosphine were established for eggs and larvae of *P. interpunctella* and eggs of *T. castaneum* using laboratory susceptible strains of the two species. For *T. castaneum* and *P. interpunctella* eggs, discriminating doses were 62.4 and 107.8 ppm, respectively, over a 3-d fumigation period, and for *P. interpunctella* larvae, discriminating dose was 98.7 ppm over a 20-h fumigation period. Discriminating dose tests on adults and eggs showed that 4 out of 11 *T. castaneum* populations tested had resistance frequencies that ranged from 42 to 100% for adults and 54 to 100% for eggs. LC_{99} values for the susceptible and the most resistant adults of *T. castaneum* were 7.4 and 356.9 ppm over 3 d, respectively. LC_{99} values for *T. castaneum* eggs were 51.5 and 653.9 ppm, respectively. Based on adult data, the most resistant *T. castaneum* beetle population was 49× more resistant than the susceptible strain. Phosphine resistance frequencies in *P. interpunctella* eggs ranged from 4 to 20%. Results show phosphine resistance is present in both species in California. Future research will investigate phosphine resistance over a wider geographic area. In addition, the history of pest management practices in facilities where insects tested in this study originated will be determined in order to develop phosphine resistance management strategies for California almond storage and processing facilities.

Key words: phosphine, stored-product, resistance management, red flour beetle, Indian meal moth

The United States is the world's leading producer of almonds. The Central Valley of California produces nearly all almonds in the United States, with annual production exceeding 840,000 metric tons valued at > $6.5bn (NASS 2015). Such high production levels are associated with stored almond postharvest pest management practices geared toward protecting this lucrative commodity from losses caused by insect and microbiological pests. Postharvest fumigation is a method of choice for disinfestation of incoming field pests and stored-product insect pests in dried fruits and nuts (Johnson et al. 2012). California almonds receive at least three fumigations before storage or marketing, that is, field disinfestation of newly harvested almonds to kill any incoming field pests, fumigation after hulling/shelling, and fumigation before marketing or storage. Phosphine or hydrogen phosphide (PH_3) is the most widely used fumigant on stored almonds; the use of sulfuryl fluoride (SF) is increasing. Because of the regulatory phase-out of methyl bromide (MeBr), the reliance on PH_3 and SF is expected to continue into the foreseeable future. However, these two important fumigants have their own set of challenges. Limitations with SF include species-specific ovicidal deficiencies and residue concerns (UNEP 2011), whereas rapid development of resistance by stored-product insect pests is a major challenge for the continued and effective use of PH_3 (Collins et al. 2001, Opit et al. 2012a).

Phosphine resistance in stored-product insects has become a global concern (Champ and Dyte 1976, Nayak et al. 2003, Benhalima et al. 2004, Opit et al. 2012a). A worldwide survey carried out by Food and Agricultural Organization (FAO) of the United Nations first reported PH_3 resistance in six species from 33 out of 82 countries surveyed (Champ and Dyte 1976). Since then, reports on presence of PH_3 resistance in stored-product insects have been documented from many countries around the world, including the United States (Zettler and Cuperus 1990, Rajendran 1999, Collins

et al. 2001, Cao et al. 2003, Benhalima et al. 2004, Lorini et al. 2007, Pimentel et al. 2010, Opit et al. 2012a, Ahmad et al. 2013, Nayak et al. 2013, Jittanun and Chongrattanameteekul 2014, Cato 2015, Chen et al. 2015, Koçak et al. 2015, Sağlam et al. 2015). Currently, 15 species of insects are known to have developed resistance to PH_3 (Champ and Dyte 1976, Chaudhry 2000, Nayak et al. 2003), which continues to expand in distribution (Benhalima et al. 2004) and the number of affected species (Pimentel et al. 2009). Leaky storage facilities and the practice of overdosing to compensate for the lack of airtight storage structures lead to fumigation failures because of under-dosing. This results in higher frequency of applications leading to a higher selection pressure for PH_3 resistance. Consequently, over time, presence of resistant pest insects combined with the selection pressure results in higher resistance frequencies in pest populations and ultimately loss of efficacy of PH_3 against resistant populations (Benhalima et al. 2004).

There is clear documented evidence of PH_3 resistance in the United States from late 1980s to date. In the late 1980s and 1990s, Zettler and others reported PH_3 resistance in the United States in *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae), *Cadra cautella* (Walker) (Lepidoptera: Pyralidae), and *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) from peanut storage facilities (Zettler et al. 1989); in *T. castaneum* and *Rhyzopertha dominica* (F.) (Coleoptera: Bostrichidae) from wheat stored in farms (Zettler and Cuperus 1990); in *T. castaneum* and *Tribolium confusum* Jacquelin du Val (Coleoptera: Tenebrionidae) from flour mills (Zettler, 1991); and in *Lasioderma serricorne* (F.) (Coleoptera: Anobiidae) from tobacco storage facilities (Zettler and Keever, 1994). Twenty years later, substantial increase in PH_3 resistance was reported in *R. dominica* and *T. castaneum* populations in Oklahoma (Opit et al. 2012a). Recently, PH_3 resistance has also been reported in *Cryptolestes ferrugineus* (Stephens) (Coleoptera: Laemophloeidae) in Oklahoma (Konemann et al. 2014, 2015). The levels of resistance of the most resistant populations of *R. dominica*, *T. castaneum*, and *C. ferrugineus* in these studies were 1,519×, 119×, and 134×, respectively, more resistant than their susceptible counterparts (Opit et al. 2012a, Konemann et al. 2015). Level of resistance is an approach researchers use to compare lethal doses using response data obtained in two bioassays, that is, a measure of relative toxicity by comparison of the concentration–response relationship. The level of resistance is the ratio of the lethal doses compared (Robertson et al. 2007). It is noteworthy that PH_3 resistance studies in the United States and worldwide have used adults or larvae (in the case of moths). However, the adult stage is usually the most fumigant susceptible life stage. The reason for using adults or larvae (moths) in PH_3 resistance surveys could be because they are easily isolated and screening tests for resistance to PH_3 could be done rapidly (Bell et al. 1977). Because eggs of stored-product insects are usually the most fumigant-tolerant life stage and require much higher concentrations of PH_3 to achieve similar level of mortality compared with other life stages (Bell 1976, Hole et al. 1976), studies that aim to develop PH_3 resistance management strategies for mitigating PH_3 resistance need to target the most fumigant-tolerant life stage.

There are currently no published studies on PH_3 resistance in stored-product insect pests in California almond storage and processing facilities. Therefore, this study was conducted to provide information on PH_3 resistance in *T. castaneum* eggs and adults and in *P. interpunctella* eggs and larvae from California almond storage and processing facilities. The first objective was to determine presence or absence of PH_3 resistance in each of the two life stages of each of these two species. There are currently no published discriminating doses for eggs and larvae of *P. interpunctella* and eggs of *T. castaneum*. Therefore, this objective involved determining the discriminating doses of PH_3 for eggs and larvae of *P. interpunctella* and eggs of *T. castaneum*. The second objective was to determine resistance frequencies of *T. castaneum* eggs and adults and *P. interpunctella* eggs and larvae. A third objective was to determine the levels of resistance found in each of the two life stages of each of the two species, in cases where resistance frequencies of >40% were found. Differences in resistance frequencies and lethal concentrations of PH_3 required to kill different life stages of the same population—for both susceptible and resistant insects—and possible implications for resistance management are discussed.

Materials and Methods

Insects

Experiments to establish resistance frequencies and levels of resistance in *T. castaneum* and *P. interpunctella* were conducted in 2014 and 2015 at the Stored Product Entomology Laboratory in the Department of Entomology and Plant Pathology, Oklahoma State University. The 11 *T. castaneum* and 6 *P. interpunctella* populations used in these studies were started using insects obtained by sampling infested almond from storage and processing facilities in the Central Valley of California in 2013 and 2014. The insects were identified by using keys from a handbook "Stored Grain Insects" (USDA 1986). In order to maintain anonymity of facilities that participated in this research, field-collected populations were given code names, for example, "Box A *Tc*" representing an *T. castaneum* population from facility "A." The code names facilitated identification of insect populations from different facilities. Laboratory susceptible strains of *T. castaneum* and *P. interpunctella* were used for the experiments to determine discriminating doses and as controls in resistance frequency bioassays and dose–response tests. Voucher specimens of 20 adult insects of each population were preserved in 95% ethanol and deposited with the K.C. Emerson Entomology Museum at Oklahoma State University under lot numbers 146 (Box B *Tc*), 147 (Box BM *Tc*), 148 (Box BN *Tc*), 149 (Box BR *Tc*), 150 (Box E3 *Tc*), 151 (Box F *Tc*), 152 (Box I *Tc*), 153 (Box N *Tc*), 154 (Box S *Tc*), 155 (Box T *Tc*), 105 (laboratory susceptible Tc), 156 (laboratory susceptible *Pi*), 157 (Box E *Pi*), 158 (Box F *Pi*), 159 (Box N *Pi*), 160 (Box O *Pi*), 161 (Box R *Pi*), and 162 (Box W *Pi*). Each sample of infested almonds received was transferred to a 946-ml glass jar, labeled, and placed in an incubator at 28 ± 1 °C and 65 ± 5% RH for 2–3 wk to allow for the immature stages to develop. Adults of *T. castaneum* and eggs of *P. interpunctella* were transferred to laboratory diet. *Tribolium castaneum* were reared on a mixture of 95% all-purpose wheat flour and 5% Brewer's yeast (wt:wt) at 28 ± 1 °C and 65 ± 5% RH, and *P. interpunctella* were reared on yellow corn meal diet at the same rearing conditions as *T. castaneum*.

Estimating Discriminating Dose

To estimate discriminating doses for eggs of *T. castaneum* and eggs and larvae of *P. interpunctella*, a procedure similar to the FAO Method No. 16 was used (FAO 1975). Based on preliminary experiments, concentrations of PH_3 required for eggs of both species ranged from 10 to 110.6 ppm over a 3-d fumigation period and that for *P. interpunctella* larvae ranged from 5.5 to 110.6 ppm over a 20-h fumigation period. These concentrations were attained by injecting pre-calculated volumes of 10,000 ppm of PH_3 gas, in 3.92-liter fumigation jars (S-12758M, Uline, Waukegan, IL; referred to as fumigation jars hereafter). The volume of 10,000 ppm of PH_3 to be

injected in each fumigation jar was calculated using a formula, $C_1V_1 = C_2V_2$, where C_1 is the starting concentration of 10,000 ppm, C_2 is the target concentration, V_2 is the 3,9200-ml volume of the fumigation jar, and V_1 is that volume of 10,000 ppm phosphine gas that is injected in each fumigation jar. The volume was then adjusted by 10% to account for any loss during the process of injection. Each concentration was replicated three times. Fumigations were conducted at 25 °C.

Obtaining Eggs

To obtain eggs for fumigation, 200–300 adult *T. castaneum* were transferred from lab culture into a 473-ml glass jar containing 20 g wheat flour as a substrate and covered with two pieces of filter paper and secured using a metal ring. For *P. interpunctella*, 100–200 adults were aspirated into a 946-ml glass jar that was covered with a wire screen (U.S. Standard #40, 0.42-cm openings) and secured with a metal ring. The jars containing adults were placed in an incubator with conditions of 28 ± 1 °C and 60 ± 5% RH. After 3 d, *T. castaneum* eggs were harvested by sifting contents of jars containing the egg-laying insects through U.S. Standard #20 and #50 (0.84- and 0.297-mm openings, respectively) pair of sieves (Seedburo Equipment Company, Des Plaines, IL). *Plodia interpunctella* eggs were harvested by passing the eggs laid inside the jar through the wire screen cover into a clean petri dish.

Preparation of Insects

For each species, fifty 0- to 3-d-old eggs were placed on a transparent piece of double-sided sticky tape that was attached to a piece of black filter paper (2 by 1 cm) using a soft paint brush. Black filter paper was used to facilitate counting white eggs because of the background contrast created. A piece of filter paper containing 50 eggs was then placed in a glass vial (29 by 65 mm) without diet. For *P. interpunctella* larvae, 50 fourth-instar larvae were selected randomly using a soft forceps and transferred to a glass vial (29 by 65 mm) containing 0.5 g of yellow corn meal diet. The mouth of each glass vial was covered with a piece of paper towel that was secured using Teflon tape (Blue Hawk Plumber's Tape). Each vial was labeled with information indicating the species, PH_3 concentration, and replication. The labeled vials were then placed inside respective fumigation jars assigned to different concentrations of PH_3.

Fumigation Jars

A fumigation jar consisted of a 3.92-liter glass jar (S-12758M, Uline, Waukegan, IL) along with a plastisol-lined metal lid (S-18023, Uline, Waukegan, IL). The lid was equipped with a port in the center, which was fitted with a rubber injection septum for introduction and sampling of the fumigant. A double layer of Teflon tape was applied to the outside of the lid after the lid was screwed on and to the outside edge of the rubber septum to prevent gas leakage. Prior to placing vials containing insects inside jars, two drops of water were added to each jar to maintain 65 ± 5% RH. After placing the vials containing eggs or larvae in fumigation jars and prior to injection of PH_3, a volume of air 1.5-times the amount of gas to be added was removed using a gas-tight syringe (100 ml, Hamilton 1100 SL SYR, Hamilton Inc., Reno, NV). Pre-calculated volumes of 10,000 ppm PH_3 were then added (injected) into fumigation jars to give the desired concentrations in the fumigation jars. PH_3 was injected through the rubber septum placed on each port.

In the determination of the discriminating doses for eggs of *T. castaneum* and *P. interpunctella*, nine different concentrations of PH_3 were evaluated, which ranged from 9.8 to 110.6 ppm over a 3-d exposure period, and those for *P. interpunctella* larvae ranged from 5.5 to 110.6 ppm over a 20-h exposure period. All experiments were conducted at 25 °C. The rationale behind choosing the 20-h exposure period was for consistency with the FAO.

PH_3 Concentration Analysis

The concentration of PH_3 gas in each fumigation jar was measured at the beginning and at the end of the respective exposure periods using a gas chromatographic-flame photometric detector (GC-FPD; Model 8610C, SRI Instruments, Torrance, CA). The GC-FPD is equipped with Rt – QS-Bond 30-m, 0.53-mm ID Silica Column. Column and detector temperature is an isothermal temperature at 200 °C. During the operation, gas flow rates were: helium carrier gas at 20 ml/min, hydrogen at 35 ml/min, and air at 20 ml/min. With these conditions, the retention time of phosphine is 2 to 3 min, depending on the concentration of the sample being analyzed. Temperature run program is 190 min. Prior to taking gas samples from each jar, gas in the jar was evenly mixed by pumping three times with a 100-ml syringe (Hamilton 1100 SL SYR, Hamilton Inc., Reno, NV). The GC-FPD was calibrated using 200 ppm PH_3 gas (Matheson Tri-gas) before taking samples from the jars. The concentrations were established using a standard curve based on 50, 40, 30, 20, and 10 µl of 200 ppm PH_3. For establishing the standard curve, gas samples corresponding to each of the above volumes were withdrawn from the Tedlar bag containing 200 ppm of PH_3 using a calibrated gas-tight syringe (50 µl, Hamilton 1705 TLL SYR, Hamilton Inc., Reno, NV) and injected into the on-column injector of the GC-FPD. The areas under the peak in microvolts (µV) counts were recorded along with the volume of PH_3 injected. PH_3 volumes were regressed against measured peak areas to generate a straight-line regression equation that had a coefficient of determination (r^2) value between 0.96 and 0.99 in all cases. Thirty microliter gas sample from each fumigation jar was analyzed using the GC-FPD and quantified using the regression equation generated from the standard curve.

Post-Fumigation Protocol

After the sampling of end concentrations, jars were aerated by removing lids inside a certified fume hood. The vials in each jar were removed and kept in a plastic box (42.9by 29.2 by 23.5 cm) in an incubator maintained at 25 ± 1 °C and 70 ± 5% RH for 14 d. For vials containing *P. interpunctella* larvae, the paper towel covering the mouth of each vial was replaced with a perforated vial cap to prevent larvae from escaping. Eggs were counted after 7 d and held for an additional 1 wk to account for any delay in egg hatch due to exposure to PH_3. Final mortality assessments for eggs and larvae were conducted after 14 d. Larvae that did not move after prodding with a blunt-end forceps were counted as dead.

Data Analysis

The experimental designs for determining discriminating doses were completely randomized designs with three replications. Phosphine dose–response mortality data for each of the life stages of each species were subjected to probit analysis using PoloPlus (LeOra Software, Petaluma, CA; LeOra Software, 2005) to estimate lethal concentrations to kill 50, 95, and 99% individuals in samples, that is, LC_{50}, LC_{95}, and LC_{99} values, and their 95% confidence intervals (CIs). The discriminating dose is expected to kill all susceptible insects (Food and Agriculture Organization 1975). The objective of this study was to establish doses to control 99% of the individuals in samples tested. Therefore, depending on the

species and life stage, the discriminating dose is the upper limit of the 95% CI of the LC_{99} value at a given exposure period at 25 °C.

Frequency of PH_3 Resistance

To determine PH_3 resistance in *T. castaneum* adults, a discriminating dose of 30 ppm of PH_3 for 20-h exposure recommended by FAO Protocol No. 16 was used (Food and Agriculture Organization 1975) on mixed-sex, 1- to 6-wk-old adult *T. castaneum* with the following modifications. For the *T. castaneum* laboratory susceptible strain and 11 field-collected populations, 50 adult insects of each species were placed in individual glass vials that contained 0.5 g of cracked wheat diet. Then, the vials containing insects were covered with a piece of paper towel that was secured using a Teflon tape and placed in each of three fumigation jars. Insects were also placed in another three fumigation jars prepared as previously described, but fumigant was not added to these jars as control.

In order to determine PH_3 resistance frequencies in *T. castaneum* and *P. interpunctella* eggs, discriminating doses estimated in current study were used (Table 1). The discriminating doses used were 62.4 and 107.8 ppm, respectively, over a 3-d exposure period at 25 °C. The procedures for vial setup and fumigation were similar to those described above for determining discriminating dose.

For *P. interpunctella* larvae, the discriminating dose tested was 98.7 ppm over a 20-h exposure period at 25 °C. Fourth-instar *P. interpunctella* larvae were selected randomly from cultures and placed in glass vials with 0.5 g of yellow corn meal diet. The vials were prepared as previously described above. At the end of 20-h fumigation, the paper towel covering of each vial was replaced with a perforated vial cap. This was done to prevent *P. interpunctella* larvae from escaping.

For all life stages and species, mortality assessments were conducted 14 d after fumigation. Eggs of both species were counted after 7 d and held for an additional 1 wk to account for any delayed hatching due to exposure to PH_3. Delay in egg hatch in *T. castaneum* as result of exposure to phosphine has been previously reported (Rajendran 2000). Any insect that survived the discriminating dose bioassay had detectable PH_3 resistance.

Levels of PH_3 Resistance

Field-Collected Populations for Testing

Populations of insects that had ≥ 40% survival in three replications, based on the discriminating dose bioassay, were tested in dose–response studies to determine the levels of resistance. Concentrations of PH_3 required to kill 50, 95, and 99% of the sample insect population (LC_{50}, LC_{95}, and LC_{99}) for each life stage of the selected populations were estimated. Based on discriminating dose bioassay results, four populations of *T. castaneum*, namely, Box B *Tc*, Box BR *Tc*, Box BM *Tc*, and Box BN *Tc*, had ≥ 40% resistance frequencies in both life stages, that is, eggs and adults (Tables 2 and 3).

Adult Insects

In the determination of the levels of resistance of the Box B *Tc* and Box BR *Tc* populations that had 42–66% survival in the discriminating dose bioassay, concentrations of PH_3 evaluated were 0.0, 3.4, 7.0, 8.3, 16.4, 31.7, 55.2, and 110.6 ppm. For populations Box BM *Tc* and Box BN *Tc* that had 88–100% survival in discriminating dose bioassay, concentrations of PH_3 evaluated were 0.0, 17.7, 34.0, 55.2, 110.6, 139.2, 212.2, and 273.1 ppm. Fifty mixed-sex adults of each of the four populations were placed in each vial as previously described above. The vials were then placed in fumigation jars as described above. In addition, 50 susceptible insects in vials were placed in each of the jars containing insects of the field populations. The exposure period was 3 d and all experiments were conducted at 25 °C. Mortality assessments were conducted 5 d post-fumigation.

Eggs

In the determination of the levels of resistance of the Box B *Tc* and Box BR *Tc* populations of *T. castaneum* that had 44-84% survival in the discrimination dose bioassay, concentrations of PH_3 evaluated were 0.0, 33.3, 53.1, 81.1, 124.6, 182.2, 238.4, 260.8, and 325.5 ppm. For populations Box BM *Tc* and Box BN *Tc* that had 100% survival in the discriminating dose bioassay, concentrations of PH_3 evaluated were 0.0, 81.1, 124.6, 182.2, 238.4, 260.8, 325.5, 465.0, and 684.0 ppm. Vial setup and fumigation procedures were as previously described above. In addition, a vial containing 50 susceptible eggs was placed in each of the jars containing eggs of the field populations. Fumigation lasted 3 d and mortality was assessed 7 d later-all eggs in the control hatched in 7 d.

Susceptible Strain

In the determination of the level of mortality for adults and eggs of the susceptible *T. castaneum*, concentrations of PH_3 evaluated for adults were 0.0, 1.9, 2.4, 3.3, 5.4, 7.1, and 7.4 ppm. The concentrations evaluated for *T. castaneum* eggs ranged from 0.0 to 110.6 ppm, as described above in determining discriminating dose. Insects for fumigation experiments were prepared as previously described above. Mortality was assessed 5 d and 7 d after fumigation for adults and eggs, respectively. Eggs were held for an additional 1 wk in order to determine any delay in egg hatch due to exposure to PH_3.

Table 1. Probit analyses of mortality for eggs of *P. interpunctella* and *T. castaneum*, and larvae of *P. interpunctella*

Species	*N*	Slope ± SE	LC_{50} (95% CI)	LC_{95} (95% CI)	LC_{99} (95% CI)	χ^2 (df) [H[a]]
Eggs						
P. interpunctella	1,720	3.9 ± 0.17	21.5 (19.8–23.3)	56.5 (49.1–67.8)	84.4 (70.0–107.8)[b]	83.0 (31) [2.7]
T. castaneum	1,466	5.4 ± 0.25	19.1 (17.6–20.5)	38.5 (34.5–44.5)	51.5 (44.6–62.4)	64.8 (25) [2.6]
Larvae						
P. interpunctella	1,348	2.86 ± 0.16	10.5 (9.2–11.7)	39.3 (32.1–51.2)	68.1 (52.1–98.7)	58.5 (25) [2.3]

Fumigation periods for eggs and larvae were 3 d and 20 h, respectively, at 25 °C. Lethal concentrations are in parts per million (ppm). Laboratory susceptible strains of *T. castaneum* and *P. interpunctella* were used for the experiments to determine discriminating doses.

[a] Heterogeneity factor, chi-square value/degrees of freedom (chi-square is significant, $P < 0.05$).

[b] Discriminating dose is the upper limit of 95% CI of LC_{99} value for each species and life stage.

G-factor, $t^2/V(b)/b^2$, where t = Student's t with error degrees of freedom, V(b) is the slope variance estimate given in the variance-covariance matrix, and b is the slope estimate; these values, for *T. castaneum* eggs, *P. interpunctella* eggs, and *P. interpunctella* larvae, 0.008, 0.074, and 0.012, respectively. G-values that are less than 0.5 suggest that the value of the mean is within the limit at 95% probability.

Table 2. Survival of *T. castaneum*, adults and eggs, from the laboratory susceptible strain and field-collected populations

Life stage	Population	Percentage survival		
		Rep 1	Rep 2	Rep 3
Adults	Box B *Tc*	48	48	46
	Box BR *Tc*	42	66	54
	Box BM *Tc*	88	92	98
	Box BN *Tc*	94	96	100
	Box E1 *Tc*	0	0	0
	Box E2 *Tc*	0	0	0
	Box F *Tc*	0	0	0
	Box I *Tc*	0	0	0
	Box N *Tc*	0	0	0
	Box S *Tc*	0	0	0
	Box T *Tc*	0	0	0
	Susceptible *Tc*	0	0	0
Eggs	Box B *Tc*	66	44	66
	Box BR *Tc*	82	84	84
	Box BM *Tc*	100	100	100
	Box BN *Tc*	100	100	100
	Box E1 *Tc*	0	0	0
	Box E2 *Tc*	0	0	0
	Box F *Tc*	0	0	0
	Box I *Tc*	0	0	0
	Box N *Tc*	0	0	0
	Box S *Tc*	0	0	0
	Box T *Tc*	0	0	0
	Susceptible *Tc*	0	0	0

Discriminating doses for adults and eggs were 30 ppm phosphine over 20-h exposure and 62.4 ppm over 3-d exposure, respectively, at 25 °C.

Table 3. Survival of *P. interpunctella*, larvae and eggs, from the laboratory susceptible strain and field-collected populations

Life stage	Population	Percentage survival		
		Rep 1	Rep 2	Rep 3
Larvae	Box E *Pi*	0	0	0
	Box F *Pi*	0	0	0
	Box N *Pi*	0	0	0
	Box O *Pi*	0	0	0
	Box R *Pi*[a]	0	0	0
	Box W *Pi*[a]	0	0	0
	Susceptible *Pi*	0	0	0
Eggs	Box E1 *Pi*	10	8	6
	Box F *Pi*	4	7	5
	Box N *Pi*	20	15	12
	Box O *Pi*	N/A[b]	N/A	N/A
	Box R *Pi*	N/A	N/A	N/A
	Box W *Pi*	N/A	N/A	N/A
	Susceptible *Pi*	0	0	0

Discriminating doses for larvae and eggs were 98.7 ppm phosphine over 20-h exposure and 107.8 ppm over 3-d exposure, respectively, at 25 °C.

[a] Only 30 larvae from each population, Box R *Pi* and Box W *Pi*, were tested because of the limited number of insects available for test.

[b] N/A Due to low numbers of *P. interpunctella* adults and difficulty in collecting sufficient (50 per treatment) eggs for resistance frequency experiments, eggs for populations Box O *Pi*, Box R *Pi*, and Box W *Pi* were not evaluated.

Data Analysis

Probit analyses of the dose–response mortality data for susceptible and resistant populations of *T. castaneum* were conducted to estimate the concentrations of PH_3 required to kill 50, 95, and 99% of individuals in susceptible and resistant populations. A ratio test to compare LCs was also conducted for eggs and adults of *T. castaneum* (Robertson et al. 2007) to determine the degree by which the field populations were more resistant to PH_3 than the susceptible laboratory strain.

Results

Discriminating Doses

Based on dose–response studies using laboratory susceptible strains, PH_3 discriminating doses for eggs of *T. castaneum* and *P. interpunctella* were 62.4 and 107.8 ppm over a 3-d fumigation period, respectively, at 25 °C. For *P. interpunctella* larvae, the discriminating dose was 98.7 ppm over a 20-h PH_3 fumigation period (Table 1). PoloPlus uses the heterogeneity factor as a correction factor when the value of Pearson's chi-square statistic (χ^2) is significant at $P = 0.05$ (LeOra software 2005). According to Finney (1952), a significantly large χ^2 value indicates that all weights have been overestimated by a factor $\chi^2/(k-2)$, where k is the number of dosages tested. Therefore, all variances should be multiplied by this heterogeneity factor as compensation for the overestimation. Despite heterogeneity values greater than 1, the respective fitted lines tracked the experimental data. Heterogeneity values >1 in our data for both species is evidence of heterogeneity in response to PH_3.

Resistance Phenotype Frequencies

Resistance phenotype frequencies of adult *T. castaneum* ranged from 42 to 100% and those of eggs ranged from 44 to 100% (Table 2). Numerically, Box BM *Tc* and Box BN *Tc* populations had the highest resistance frequencies, 88–100% in adults and 100% in eggs. Phenotype resistance frequencies of *P. interpunctella* eggs ranged from 4 to 20%, but none of the larvae from the field populations survived the discriminating dose bioassay (Table 3), indicating lack of detectable resistance in these larvae. Insect mortality in control jars that did not receive fumigant was extremely low (<2%).

Levels of Resistance

For *T. castaneum* adults, the concentrations of PH_3 required to kill 50, 95, and 99% of the susceptible *Tc* strain and Box B *Tc*, Box BR *Tc*, Box BM *Tc*, and Box BN *Tc* populations were estimated (Table 4) and compared (Table 5). In all cases, the comparisons yielded 95% CIs that did not include 1, indicating that the LC values required to attain the referenced mortality in resistant populations in each case were different from the LC value for the susceptible strain (Table 5). Based on the LC_{99} comparisons for adults, the levels of resistance in populations Box B *Tc*, Box BR *Tc*, Box BM *Tc*, and Box BN *Tc* were 6.8, 7.4, 40.1, and 48.5 times, respectively. For eggs, the levels of resistance for referenced four populations were 4.3, 5.5, 11.8, and 12.7, respectively. Correlation value for the levels of resistance between eggs and adults of resistant *T. castaneum* populations was high ($r = 0.99$, $P < 0.0001$).

Table 4. Probit analyses of mortality for the laboratory susceptible strain and phosphine-resistant field populations of *T. castaneum* adults and eggs, after 3 d exposure to phosphine concentrations at 25 °C

Populations	N	Slope ± SE	LC_{50} (95% CI)	LC_{95} (95% CI)	LC_{99} (95% CI)	χ^2 (df) [H[a]]
Adults						
Susceptible *Tc*	901	6.9 ± 0.37	3.4 (3.3–3.6)	5.9 (5.5–6.4)	7.4 (6.8–8.2)	12.9 (16) [0.8]
Box B *Tc*	1,060	3.0 ± 0.18	8.6 (7.9–9.3)	29.9 (26.0–35.6)	50.2 (41.6–63.5)	16.9 (19) [0.9]
Box BR *Tc*	1,058	3.3 ± 0.18	10.5 (9.7–11.3)	33.5 (29.3–39.6)	54.3 (45.4–67.6)	17.1 (19) [0.9]
Box BM *Tc*	904	2.5 ± 0.16	34.4 (29.9–39.0)	157.3 (129.5–202.4)	295.2 (226.0–421.3)	20.9 (16) [1.3]
Box BN *Tc*	905	2.4 ± 0.15	39.4 (34.4–44.6)	187.2 (152.7–243.7)	356.9 (270.4–515.9)	21.5 (16) [1.4]
Eggs						
Susceptible *Tc*	1,243	5.4 ± 0.25	19.2 (16.7–21.6)	38.2 (32.2–50.1)	51.5 (44.6–62.4)	64.8 (25) [2.6]
Box B *Tc*	1,665	3.2 ± 0.18	41.5 (36.5–46.2)	135.1 (120.3–155.9)	220.4 (187.1–272.1)	31.3 (22) [1.4]
Box BR *Tc*	1,817	3.4 ± 0.15	56.5 (50.9–62.0)	175.2 (154.9–203.9)	279.9 (236.6–346.7)	45.6 (22) [2.1]
Box BM *Tc*	1,802	3.6 ± 0.17	137.4 (126.5–147.5)	392.1 (356.3–440.9)	605.5 (527.9–719.4)	32.6 (22) [1.5]
Box BN *Tc*	1,801	3.4 ± 0.18	137.6 (129.2–145.5)	414.2 (381.3–456.8)	653.9 (580.3–755.1)	20.4 (22) [0.9]

Lethal concentration values (LC) are in parts per million (ppm).

[a] Heterogeneity factor, chi-square value/degrees of freedom.

P-values for *T. castaneum* adults, namely, Susceptible *Tc*, Box B *Tc*, Box BR *Tc*, Box BM *Tc*, and Box BN *Tc* are 0.68, 0.57, 0.58, 0.18, and 0.16, respectively. For *T. castaneum* eggs, *P*-values for these populations were < 0.05, 0.09, <0.05, 0.06, and 0.56, respectively.

G-factor, $t^2/V(b)/b^2$, where t = Student's t with error degrees of freedom, V(b) is the slope variance estimate given in the variance-covariance matrix, and b is the slope estimate; these values, for *T. castaneum* adults, namely, Susceptible *Tc*, Box B *Tc*, Box BR *Tc*, Box BM *Tc*, and Box BN *Tc* are 0.011, 0.013, 0.012, 0.015, and 0.014, respectively. For *T. castaneum* eggs, g factor values for referenced populations were 0.012, 0.012, 0.007, 0.09, and 0.009, respectively. G-values that are less than 0.5 suggest that the value of the mean is within the limit at 95% probability.

Table 5. Comparison of lethal concentrations (ppm) required to kill 50, 95, and 99% of *T. castaneum* adults and eggs of the susceptible strain and field-collected populations, after 3-d exposure to phosphine concentrations at 25 °C

Populations compared	Lethal concentration ratios		
	LC_{50} (95% CI)	LC_{95} (95% CI)	LC_{99} (95% CI)[a]
Adults			
Box B *Tc* vs susceptible *Tc*	2.5 (2.3–2.8)	5.1 (4.3–6.1)	6.8 (5.4–8.6)
Box BR *Tc* vs susceptible *Tc*	3.1 (2.8–3.4)	5.7 (4.8–6.7)	7.4 (5.9–9.2)
Box BM *Tc* vs susceptible *Tc*	10.1 (9.0–11.4)	26.8 (22.1–32.5)	40.1 (30.7–52.4)
Box BN *Tc* vs susceptible *Tc*	11.6 (10.4–12.9)	31.9 (26.1–38.9)	48.5 (36.9–63.7)
Eggs			
Box B *Tc* vs susceptible *Tc*	2.2 (1.9–2.4)	3.5 (3.1–4.0)	4.3 (3.6–5.1)
Box BR *Tc* vs susceptible *Tc*	2.9 (2.7–3.2)	4.6 (4.1–5.2)	5.5 (4.7–6.5)
Box BM *Tc* vs susceptible *Tc*	7.2 (6.7–7.8)	10.2 (9.1–11.4)	11.8 (10.1–13.7)
Box BN *Tc* vs susceptible *Tc*	7.2 (6.7–7.8)	10.8 (9.6–12.1)	12.7 (10.8–15.0)

[a] A 95% confidence interval that includes 1 in any comparison means that the two lethal concentrations are not significantly different.

Discussion

Discriminating doses for immature life stages of *T. castaneum* and *P. interpunctella* established in this study can be used in future PH_3 resistance surveys involving immature life stages of these two species. Some researchers have previously conducted PH_3 resistance studies on immature life stages of stored-product insect pests (Bell et al. 1977, Zettler et al. 1989). However, discriminating doses that could be used for PH_3 resistance surveys for immature life stages were not developed. In addition, the protocol for establishing discriminating doses described in this study can also be used as a reference for other stored-product insect species.

Discriminating dose bioassays using the respective discriminating doses for eggs and larvae of *P. interpunctella* showed that PH_3 resistance was only present in eggs but not in larvae, and resistance frequencies in eggs ranged from 4–20%. Zettler *et al.* (1989) reported 2–24% resistance frequencies in *P. interpunctella* larvae collected from peanut storage facilities in 1989. The discriminating dose used in that study was 10 ppm of PH_3 during 5-d exposure at 27 ± 3 °C, whereas in this study, the discriminating dose for fourth-instar *P. interpunctella* larvae was 98.7 ppm over a 20-h exposure period.

For *T. castaneum*, we found clear evidence of resistance to PH_3 in both eggs and adults of 4 out of 11 field-collected populations. Correlation value for resistance frequencies at egg and adult stage was high ($r = 0.98$); that is, populations that had high resistance frequencies in adults also had high resistant frequencies in eggs and vice-versa. It is also important to note that resistance frequencies for eggs were numerically greater than those for adults, for all 11 populations. In 1977, Bell et al., in a similar study to evaluate resistance frequencies in eggs and adults of *R. dominica*, reported that there was correlation between resistance in the adult and egg stages.

Bell et al. (1977) concluded that the FAO discriminating dose test on adults was an effective way of identifying resistant strains. The discriminating dose for *R. dominica* eggs used in their study was 114.3 ppm over a 24-h fumigation period. However, these researchers only determined presence–absence of resistance in the egg stage of insects that were resistant as adults and did not determine frequencies of resistance. The Bell et al. (1977) PH_3 discriminating dose for *R. dominica* eggs (114.3 ppm over 24-h exposure) and the discriminating dose from this study (62.4 ppm over a 3-d exposure) indicate that discriminating doses for eggs are much higher than those for adults (or larvae); this also confirms findings from other studies that show that eggs of stored-product insects are usually the most fumigant tolerant life stage (Bell 1976, Hole et al. 1976).

As mentioned above, PH_3 resistance in *T. castaneum* from wheat storage facilities has previously been reported in Oklahoma (Zettler and Cuperus 1990, Opit et al. 2012a). Zettler and Cuperus (1990) reported one out of eight populations of *T. castaneum* tested was resistant to PH_3 and the resistance frequencies ranged from 0 to 6%. In 2012, Opit et al. (2012a) reported that eight out of nine populations of *T. castaneum* tested were resistant to PH_3 and the resistance frequencies in these populations ranged from 0 to 92%. Although these two studies, conducted 21 years apart, tested populations collected from different locations, there is a trend to more resistant populations with higher resistance frequencies in the latter study. In our current study, using insects from almond storage and processing facilities in California, 4 out of 11 *T. castaneum* populations tested were resistant to PH_3 and resistance frequencies ranged from 42 to 100%. Unlike Oklahoma populations, where a majority of the samples were resistant, we found that 64% of the populations tested in our study were completely susceptible; that is, no resistance was detected or present in both the egg and adult life stages tested. This could be because of the differences in insect pest management practices used by the wheat industry in Oklahoma and the almond industry in California. Fumigation using PH_3 is the main method of controlling insect infestations in stored wheat in Oklahoma. The recommended dosage of PH_3 for stored grain in the United States is 200 ppm over a 4-d or longer exposure at 20–30 °C and commercial grain (wheat) storage facilities fumigate 3 times per year (Cuperus et al. 1990, Leesch et al. 1995, Phillips et al. 2012). On the other hand, for the almond industry, the recommended dose is 500–1,000 ppm for a minimum of 3 d, but 5–7 d are highly recommended, at 20–30 °C. Usually almonds are fumigated over very long exposures (7 d or more), depending on whether it is a stockpile (a large accumulated stock of almonds before processing) fumigation, or fumigation of almond kernels in wooden bins stored in warehouses. However, sorption by commodity and escape from leaky storage structures may lead to significant drop of target concentrations after a couple of days depending on the commodity (in shell or shelled almonds) and storage structure (stockpile under the tarp, almond kernels stored in storage bins or in a warehouse). According to Reddy et al. (2007), sorption of PH_3 in in-shell almonds fumigated with 1,428 ppm (2 g/m^3) PH_3 was 100% at the end of 7 d. In such cases, target insect pests would be exposed to sublethal dose of PH_3, and the populations present at the time of fumigation could be selected for resistance if resistance genes were present. It is very likely that sorption and/or leakage of PH_3 that is not compensated for may have contributed to the development of resistance by selecting from heterozygous insect populations in almond storage and processing facilities.

Genetic studies in *T. castaneum* and *R. dominica* by Collins et al. (2002), Jagadeesan et al. (2012), and Schlipalius et al. (2002, 2008) have shown that two genes, *rph1* and *rph2*, are responsible for "weak" and "strong" PH_3 resistance. The *rph1* locus codes for the "weak" resistance phenotype providing moderate resistance to phosphine, whereas *rph2* by itself confers only very low-level resistance. However, when an individual is homozygous for resistance alleles at *rph1* and is either heterozygous or homozygous for *rph2*, the two loci may act synergistically to yield a strong resistance phenotype of many 100s-fold resistance compared with susceptible strain. When the same individual has both *rph1* and *rph2*, it has a much higher level of resistance known as the "strong" resistance phenotype due to the synergistic effect of *rph1* and *rph2*. Chen et al. (2015) reported that R-allele frequencies and percent resistance were highly co-related. These findings suggest that resistant individuals in heterozygous populations can be selected for strong resistance with continued phosphine use.

SF is another popular choice for postharvest pest management in almonds. After the phase-out of methyl bromide in 2005, dried fruit and nut industries have transitioned to using SF, especially, when rapid throughput fumigation is required during the peak harvest season (Gautam 2013). Because SF has a different mode of action and there is no known resistance to SF (Thoms and Phillips 2004), use of SF can control PH_3-resistant insects (Opit et al. 2016). In fact, a recent study evaluating the efficacy of SF against highly PH_3-resistant *R. dominica* and *T. castaneum* showed that SF is effective against all life stages of highly PH_3-resistant beetles (Opit et al. 2016). According to Opit et al. (2016), SF is an effective tool for PH_3 resistance management. It is likely that in populations of *T. castaneum* and *P. interpunctella* where PH_3 resistance was not present in this study, the almond storage facilities these insects originated from may be using SF for postharvest pest management. Therefore, PH_3-resistant insects have been effectively controlled.

Based on our data, the concentration of PH_3 required to achieve 99% mortality of the most resistant *T. castaneum* was 356.9 (adults) and 649.5 ppm (eggs) over a 3-d exposure period. Adults in the most resistant *T. castaneum* population in the Opit et al. (2012a) study required 377.5 ppm of PH_3 over a 3-d fumigation period for 99% mortality to be attained. We also showed that eggs require a higher PH_3 concentration to achieve a similar level of mortality. For example, LC_{99} values for eggs and adults of the susceptible and the most PH_3-resistant *T. castaneum* populations were 51.5 and 7.4, and 653.9 and 356.9 ppm, respectively, over a 3-d fumigation period. Despite the low number of populations tested, levels of resistance in eggs and adults were highly correlated ($r = 0.99$). For the effective control of both susceptible and resistant insects, it is important that the concentrations that control the most fumigant tolerant life stage, that is, eggs, of the most PH_3-resistant population should be applied. The facility managers or fumigation practitioners need to practice efficient fumigation by ensuring that storage structures under fumigation are nearly airtight (properly sealed), the recommended fumigation dosage is followed, gas concentrations during fumigation are monitored, and any loss due to sorption or leakage during fumigation is compensated for by adding more fumigant. Information on resistance frequencies and concentrations of PH_3 required to kill PH_3-resistant and PH_3-susceptible populations allow facility managers to mitigate PH_3 resistance and also delay the development of PH_3 resistance where it has not occurred. Development of rapid detection methods of phosphine resistance is also very crucial for phosphine resistance management.

Development of PH_3 resistance management strategies involves finding ways to significantly reduce the populations of PH_3-resistant insects in order to maintain a high proportion of susceptible insects in pest populations, so that PH_3 can remain a viable tool for pest management (Opit et al. 2012b). These strategies involve: 1)

delaying the development of resistance to PH_3 where it has not occurred, for example, by infrequent use of PH_3; 2) significant reduction in the population of resistant insects where PH_3 resistance already exists—in cases where resistant insects can be controlled with PH_3 concentrations still within the label-limit, a one-time high-dose fumigation to significantly reduce the population of resistant insects can be implemented; and 3) where extremely high levels of resistance are present, PH_3 resistance management can be accomplished using effective alternative methods of pest management that significantly reduce the numbers of resistant insects, for example, using integrated pest management with SF or effective protectants as tools and withholding use of PH_3 long enough to mitigate resistance. Sulfuryl fluoride, spinosad, chlorpyrifos methyl + deltamethrin, inert dusts, aeration, sanitation, and heat are all effective against PH_3-resistant insects (Cao et al. 2010, Opit et al. 2012b, Bajracharya et al. 2013, Opit et al. 2016).

This study has confirmed that PH_3 resistance is present in *T. castaneum* and *P. interpunctella* populations in California almond storage and processing facilities. The variation in PH_3 resistance observed in *T. castaneum* and *P. interpunctella* in this study suggests that more populations of these species from a broader geographic area of the Central Valley of California need to be evaluated for resistance. Future research will be aimed at determining PH_3 resistance in many more populations of *T. castaneum*, *P. interpunctella*, and other species of stored-product insects and conducting a survey of the history of pest management practices in facilities where insects tested in this study originated in order to develop PH_3 resistance management strategies for California almond storage and processing facilities. Such a survey will elucidate factors contributing to development of PH_3 resistance in insects in some facilities and those mitigating resistance development in other facilities.

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