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Phosphine Induced Abnormalities in Carbohydrate Metabolism and Macromolecular Concentrations of *Trogoderma granarium* Everts Over Various Exposure Periods

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FRS and SSA designed and supervised the project. TR performed experiments, analyzed the data and wrote first draft of the manuscript. FRS, SSA and MAS critically reviewed and approved the manuscript.

Key words
***Trogoderma granarium*, Phosphine, Carbohydrate metabolism, Macromolecules, Exposure period**

ABSTRACT

Toxic effect of LC_{20} of phosphine on carbohydrate metabolism and macromolecular concentrations in two larval instars (4^{th} and 6^{th}) of the khapra beetle, *Trogoderma granarium*, at different exposure periods (24-120 h) was determined. Two populations of khapra beetle used in this research possessed different levels of susceptibility to phosphine. Based on LC_{50} one population was termed as a susceptible population (never exposed to phosphine previously) while the other was a tolerant population (having a history of phosphine fumigation for 13 years). The LC_{50} of 4^{th} larval instar of susceptible and tolerant populations was 6 and 9 ppm while for 6^{th} larval instar it was 5.4 and 8.8 ppm, respectively. The phosphine concentration at LC_{50} value caused about 100 percent mortality when exposure period was prolonged to 120 h. The trehalose, glycogen, lipids and nucleic acid contents of both larval instars of these populations were significantly reduced, while trehalase and amylase activities were significantly increased in larvae of both populations throughout the phosphine exposure period with reference to their respective unexposed group values. In case of soluble proteins, an elevation was observed which was followed by a decline after 72 h. Similarly, free amino acid contents showed increasing trend in both larval instars of tested populations throughout the exposure period with the exception of the 4^{th} larval instars of susceptible population which decreased after 72 h. The concentration of glucose showed significant increase in 6^{th} larval instars of both populations at each exposure period but in 4^{th} larval instars it started to decrease after 72 h exposure. These phosphine induced metabolic alterations over varying exposure intervals may be helpful in controlling this insect pest by monitoring period of fumigant exposure.

INTRODUCTION

Trogoderma granarium Everts (Coleoptera: Dermestidae), commonly called khapra beetle, is one of the most destructive insect pests of wheat and many other amylaceous commodities throughout the globe, and it can also infest with great ease an extremely wide variety of durable stored products (EPPO, 2015; Athanassiou *et al*., 2016, 2019). This species is able to build rapidly high population densities, as long as the prevailing conditions are suitable for its growth and development (Hadaway, 1956; Lindgren and Vincent, 1959; Athanassiou *et al*., 2016, 2019). In a recent study, Athanassiou *et al*. (2016) reported that 20 g of wheat that contained an initial population of 20 larvae of *T. granarium* had more than 1000 individuals of this species 60 d later. Apart from its capacity to cause considerable levels of infestations, *T. granarium* is an important quarantine species in many parts of the world, such as the United States, Canada and Australia (Lowe *et al*., 2000; Myers and Hagstrum, 2012). This insect pest is native to India but now has spread in many countries through international trade and mobility (Athanassiou *et al*., 2019). Its longevity and invasion is also due to its potential to undergo long diapauses under unfavorable conditions and make additional molts (Athanassiou *et al*., 2019). Different methods of control such as plant materials and their extracts (Prakash and Rao, 2006; Gandhi and Pillai, 2011), contact insecticides like organophosphates and synthetic pyrethroids (Athanassiou *et al*., 2015) and fumigants such as phosphine and methyl bromide (Atkinson *et al*., 2004; Wang *et al*., 2006) have been evaluated with different outcomes (Haq *et al*., 2005).

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Among chemical control measures, phosphine is currently considered as the most important active ingredient to combat stored product insect pests, including *T. granarium*, and it can control, if used properly, all life stages at a relative short interval, without leaving considerable residues on the commodity. It can rapidly diffuse in the air so does not require any recirculation system during grain fumigation (Chaudhry, 2000). The larvae can easily hide in crevice, cracks and other refugee places, so phosphine has the advantage to penetrate into intergranular spaces of grains and other commodities to exert a quick toxic action. However, it may leave toxic residues on the treated commodities (Redlinger *et al*., 1979).

Despite of its effectiveness, the continuous and often improper use of phosphine lead to the evolving mechanism of resistance in major stored product insect pests, including *T. granarium* (Benhalima *et al*., 2004; Athanassiou *et al*., 2019). The development of resistance across the globe as reported by many researchers has become a serious issue and control strategies for several stored-product insect pests are failed due to the evolving status of resistance (Collins *et al*., 2005; Pimentel *et al*., 2009). Earlier reports also documented that populations of *T. granarium* have become resistant to phosphine (Bell *et al*., 1984; Bell and Wilson, 1995; Alam *et al*., 1999; Bartholomaeus and Haritos, 2005; Collins *et al*., 2005; Ahmedani *et al*., 2007; Park *et al*., 2008).

Earlier reports also revealed that fumigant exposure period is a very crucial in pest eradication strategies. (Hole *et al*., 1976) reported that long exposure period to lower concentration poses more effect than shorter exposure period at higher concentration. (Bell *et al*., 1984) suggested that phosphine exposure period should be 7 and 14 days at 20 and 30°C respectively to control susceptible and tolerant populations of khapra beetle. Similarly, Farooq and Muhammad (2000) and Sarfraz *et al*. (2000) also suggested that exposure period is also an important parameter for insect control because there are potential biochemical changes that may occur in insect pest during and after exposure to fumigant. Keeping all these reports in view, the present study was undertaken to determine the exposure period at which 100% mortality may occur. Moreover, energy reserve to fight the fumigant stress for prolonged exposure should be analyzed by measuring contents of soluble proteins, sugar and lipids and the level of some carbohydrate metabolizing enzymes. Athanassiou *et al*. (2015) and Kavallieratos *et al*. (2016) reported that larvae of Khapra beetle were more tolerant to commonly used insecticides. So, the level of above mentioned macromolecules in 4th and 6th larval instars were also evaluated after wide range of exposure to LC_{20} of phosphine. Based on the above, our goal was to propose a judicious control strategy for *T. granarium*, given that the key biochemical parameters for its control are poorly understood.

MATERIALS AND METHODS

Rearing and maintenance of larvae

Two populations of khapra beetles used in this study had different levels of susceptibility to phosphine. Based on LC_{50} one population obtained from insectary of Punjab University, Lahore was termed as susceptible population (never exposed to phosphine previously) while the other collected from Government go downs of Gujranwala was phosphine-tolerant population (having a history of phosphine fumigation for 13 years). Both populations of khapra beetle were fed on flour, whole and crushed wheat in 300ml glass jars in complete darkness. The 24 h old adults were transferred to new jars for a week to ensure egg laying. Dead beetles were discarded from the culture medium and flour containing eggs was transferred to other labelled jars. These eggs were allowed to develop into larvae, pupae and finally adult beetles. This whole procedure was repeated twice to obtain age wise homogeneous culture of khapra beetle (Riaz *et al*., 2016).

Phosphine generation and administration

Phosphine gas was evolved in fume hood from aluminium phosphide pellets (0.2g) over the surface of 10 percent (v/v) aqueous H_2SO_4 and different concentrations (ranging between 0.5 and 10 ppm, in scale of 0.5) of phosphine were calculated using the procedure mentioned in FAO (1975). The 4th and 6th instar larvae (20 insects from each) were placed in each vial in three replicates for each concentration. The calculated dose volumes for each concentration were withdrawn and injected through sterilized Hamilton micro syringe into the appropriate vacuumed glass desiccators containing vials. Same procedure was followed for control desiccators but they were kept unfumigated. These desiccators were kept at 35±1°C in temperature controlled room for 20 h. After 20 h exposure, insects were ventilated in culture medium for 48 h at 30±1°C and 60±5% r.h in continuous darkness. The response of insects under stereomicroscope after touching with brush were checked and insects showing no movement were considered dead and their number was counted. There was no mortality in control desiccators, so there was no need to calculate corrected mortality. The LC_{50} value for 4th and 6th larval instars was calculated according to Finney (1971) using Minitab 16 (Minitab, 2010) (State College, PA: Minitab, Inc).

Effect of LC_{20} on carbohydrate metabolism and metabolites over various exposure periods

Approximately, 1000 larvae from each larval instars of each population were exposed separately to its respective LC_{20} for 24, 48, 72, 96 and 120 h (Riaz *et al*., 2016). Control desiccators were also prepared in the same way as discussed above. After exposure to LC_{20}, the 4th and 6th instar larvae of both populations were evaluated for the effect of phosphine on carbohydrate metabolism and macromolecular concentrations on daily basis for five days.

Biochemical analysis

Thirty individuals from each larval instars of both populations were weighed and homogenized separately at 4°C in 1.5ml saline (0.89%) with the help of motor-driven Teflon glass homogenizer. They were centrifuged at 3000×g for 30 min at 4°C in refrigerated centrifuge. The supernatants thus obtained were used for the estimation of glucose contents by the o-toluidine method as described by Hartel *et al*. (1969). For trehalase and amylase activities the procedures of Dahlqvist (1966) and Wootton and Freeman (1982) were adopted, respectively. Trehalose contents were estimated by the anthrone method described by Carroll *et al*. (1956). Glycogen contents were extracted by macerating the larvae in KOH (30%) and estimated by the anthrone method described by Consolazio and Iacono (1963). Soluble protein contents were estimated according to Lowry *et al*. (1951). Total lipids, free amino acids and nucleic acids contents were estimated from ethanol extract of treated and control insects. For total lipids, free amino acids and nucleic acids estimation the method of Zollner and Kirsch (1962), Moore and Stein (1954) and Schneider (1957) were adopted, respectively.

Statistical analysis

Statistical analysis was done in Minitab 16 (State College, PA: Minitab, Inc) (Minitab, 2010). The data concerning the effects of LC_{20} on carbohydrate metabolism and macromolecular concentrations were compared through one way ANOVA at 0.05% probability.

RESULTS

Estimation of LC_{50}

The LC_{50} of 4th larval instar of susceptible and tolerant populations were 6 and 9 ppm while for 6th larval instar was 5.4 and 8.8 ppm, respectively.

The 6th larval instar of both populations showed 100% mortality at 96 h exposure while 4th larval instar showed approximately 100% mortality at 120 h of exposure to LC_{50} value of phosphine (Table I).

The effect of LC_{20} on carbohydrate metabolism and other macromolecules are described below.

Soluble protein contents

It was observed that soluble protein contents in unexposed groups of 4th instar larvae of both populations were increased after each time interval but exposed groups of these populations showed elevation at 24 and 48h but later on these contents started to decline. Moreover, the susceptible population possessed more soluble protein contents than the tolerant population. These contents in 4th instar larvae of susceptible and tolerant populations were significantly increased by (38, 88 and 36%) and (63, 50 and 116%) respectively after exposure to phosphine for 24, 48 and 72 h with respect to their unexposed group

Table I. Effect of phosphine administered at LC_{50} for different time periods on mortality of 4th and 6th larval instars of khapra beetle.

Strains	LC_{50} (ppm)	Exposure period				
		24 h	48 h	72 h	96 h	120 h
4th larval instars						
Tolerant	9.0	*38±0.57^{a}	42.50±0.54^{b}	51.54±0.56^{c}	81.54±0.58^{d}	99.57±0.53^{e}
Susceptible	6.0	40±1.20^{a}	55.50±0.53^{b}	68.54±0.54^{c}	84.69±0.58^{d}	100±0.0^{e}
6th larval instars						
Tolerant	8.8	41±0.52^{a}	65.11±0.57^{b}	82.22±0.55^{c}	100±0.0^{d}	100±0.0^{d}
Susceptible	5.4	43±0.53^{a}	78.50±0.51^{b}	91.21±0.0^{c}	100±0.0^{c}	100±0.0^{c}

* Mean ± standard error of mean, Means followed by the different letter in each row for each exposure time are significant from each other at 5% probability level with 95% confidence limit (Tukey's Test).

Table II. Effect of phosphine administered at LC_{20} for different time periods on carbohydrate metabolism and macromolecular concentration of 4th instar larvae of susceptible and tolerant populations of *T. granarium*.

Parameters	Exposure periods (h)	Control		Treated	
		Susceptible strain (n=20)	Tolerant strain (n=20)	Susceptible strain (n=20)	Tolerant strain (n=20)
Soluble proteins (µg/mg)	24	14.69±0.4[a]	12.25±0.93	20.29±0.45*	20.63±1.17*
	48	16.01±0.43	15.50±0.43	30.19±0.43*	23.25±0.84*
	72	18.65±0.43	15.75±0.45	25.41±0.6*	34.01±0.75*
	96	22.77±0.41	15.87±0.46	21.78±0.41*	14.25±0.48
	120	24.09±0.53	16.50±0.55	18.32±0.42*	13.89±0.25
Free Amino Acids (µg/mg)	24	46.97±0.48	44.76±0.47	108.61±0.44*	132.10±0.46*
	48	56.71±0.52	54.11±0.41	133.07±0.41*	146.78±0.45*
	72	64.97±0.41	63.66±0.42	90.02±0.48*	124.76±0.64*
	96	73.97±0.48	72.06±0.47	66.53±0.46*	96.14±0.42*
	120	70.97±0.40	65.76±0.41	36.21±0.63*	68.25±0.63*
Glucose (µg/mg)	24	75.61±0.42	32.40±0.44	119.58±0.42*	94.32±0.59*
	48	80.61±0.42	44.08±0.23	125.71±0.52*	123.26±0.59*
	72	84.88±0.44	53.11±0.47	102.62±0.40*	159.14±0.49*
	96	88.74±0.52	64.10±0.48	85.64±0.54	115.16±0.48*
	120	82.75±0.51	60.25±0.42	39.35±0.44*	57.87±0.47*
Glycogen (µg/mg)	24	1.29±0.09	1.06±0.02	1.00±0.02*	0.93±0.01*
	48	0.99±0.01	1.00±0.01	0.77±0.03*	0.84±0.01*
	72	0.93±0.06	0.94±0.01	0.65±0.04*	0.62±0.01*
	96	0.88±0.07	0.88±0.01	0.41±0.04*	0.39±0.02*
	120	0.84±0.04	0.82±0.01	0.18±0.08*	0.22±0.01*
Lipids (µg/mg)	24	0.17±0.02	0.05±0.002	0.16±0.02	0.043±0.01*
	48	0.13±0.03	0.05±0.005	0.12±0.02	0.039±0.02*
	72	0.11±0.04	0.03±0.000	0.09±0.00*	0.020±0.01*
	96	0.10±0.01	0.02±0.001	0.09±0.01*	0.014±0.01*
	120	0.09±0.01	0.01±0.001	0.06±0.00*	0.008±0.01*
DNA (µg/mg)	24	0.04±0.05	0.05±0.001	0.04±0.00*	0.04±0.01*
	48	0.04±0.06	0.04±0.001	0.03±0.01*	0.03±0.01*
	72	0.04±0.01	0.04±0.001	0.03±0.01*	0.03±0.01*
	96	0.03±0.01	0.04±0.001	0.02±0.01*	0.02±0.00*
	120	0.03±0.01	0.03±0.001	0.01±0.01*	0.02±0.00*
RNA (µg/mg)	24	0.11±0.03	0.11±0.005	0.083±0.01*	0.09±0.01*
	48	0.1±0.01	0.097±0.001	0.073±0.01*	0.08±0.01*
	72	0.09±0.01	0.09±0.001	0.06±0.001*	0.06±0.00*
	96	0.09±0.02	0.08±0.001	0.04±0.001*	0.05±0.00*
	120	0.08±0.00	0.08±0.001	0.06±0.02*	0.04±0.01*
Trehalose (µg/mg)	24	9.92±0.03	7.6±0.003	6.53±0.03	7.83±0.03
	48	10.3±0.03	12.23±0.003	6.12±0.03	7.97±0.03
	72	10.84±0.03	14.73±0.003	5.82±0.03	8.61±0.03
	96	11.01±0.03	12.32±0.003	5.66±0.03	6.26±0.03
	120	11.19±0.03	11.73±0.003	5.34±0.03	5.59±0.03
Trehalase (iu/mg)	24	0.05±0.02	0.123±0.01	0.08±0.01*	0.15±0.01*
	48	0.06±0.01	0.14±0.001	0.087±0.01	0.19±0.01
	72	0.06±0.01	0.138±0.001	0.10±0.01	0.21±0.01
	96	0.07±0.01	0.14±0.001	0.105±0.01	0.22±0.01
	120	0.08±0.01	0.14±0.001	0.11±0.01	0.20±0.01
Amylase (iu/mg)	24	0.04±0.01	0.11±0.001	0.05±0.01	0.12±0.01
	48	0.06±0.01	0.12±0.001	0.07±0.01	0.14±0.01
	72	0.06±0.01	0.12±0.001	0.12±0.01	0.17±0.01
	96	0.06±0.01	0.12±0.001	0.09±0.01	0.18±0.01
	120	0.06±0.04	0.13±0.000	0.09±0.001*	0.19±0.01*

Mean±SEM; * in each table indicates treated group value is statistically significant from control at $^{*}P<0.050$.

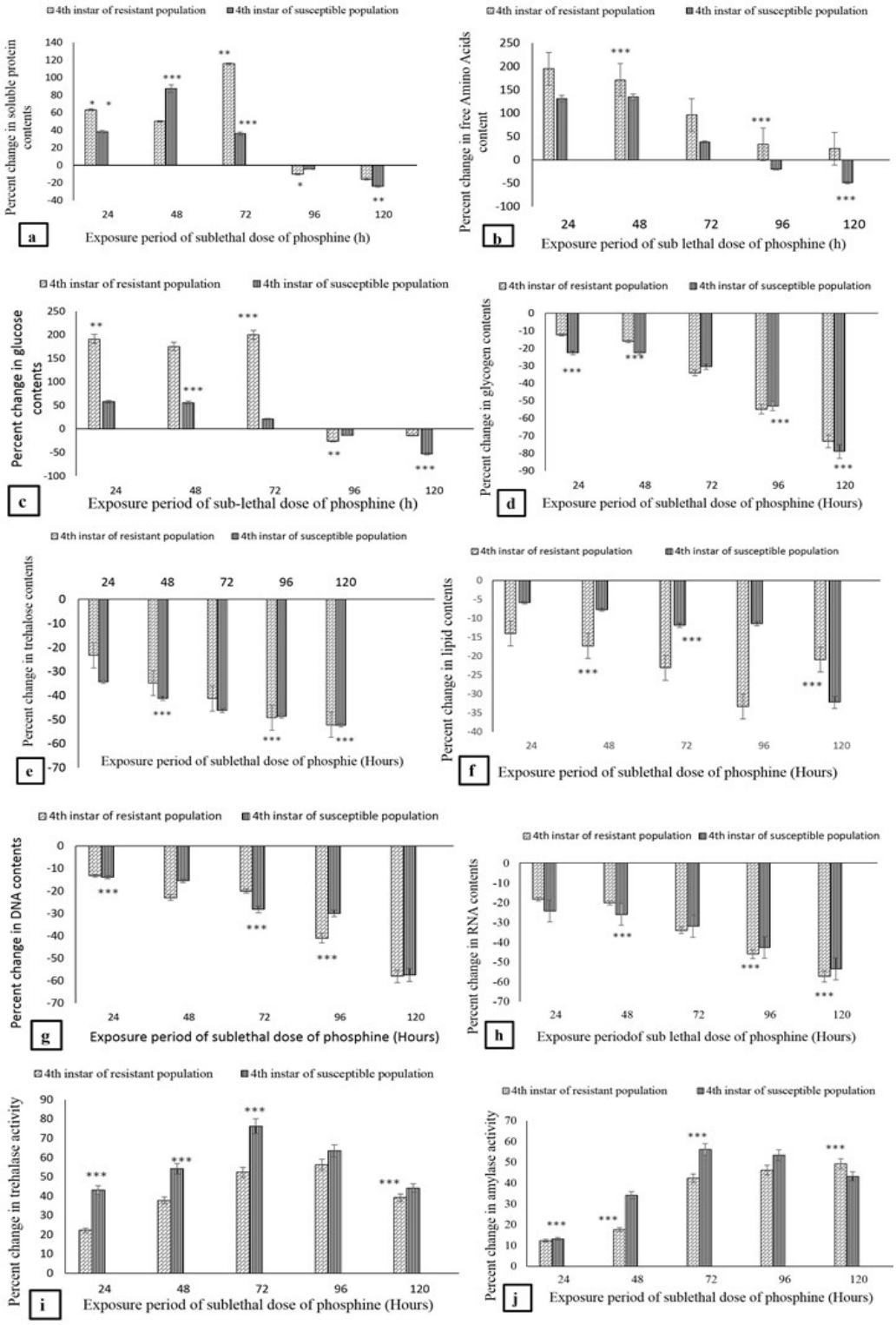

Fig. 1. Effect of phosphine administered at LC_{20} for different time periods on the percent changes in various biochemical parameters (a-j) with reference to unexposed groups of 4th instar larvae of susceptible and tolerant populations of *T. granarium*. One way ANOVA $^{*}P<0.05$; $^{**}P<0.01$ and $^{***}P<0.001$.

values. Later on, these contents were significantly reduced when exposed to phosphine for 96 and 120 h with respect to their unexposed group figure (Table II, Fig. 1a).

From the data of 6th instar larvae it was revealed that soluble protein contents in unexposed group of 6th instar larvae of both populations were increased at each interval with exception of 120 h where these contents were decreased in susceptible population. Similarly, the tolerant population presented a rise in soluble protein contents in 6th instar larvae after each exposure period but in susceptible population concentration of these contents were decreased after 72 h exposure period. The tolerant population exhibited a significant percent increase in soluble protein contents in 6th instar larvae after each exposure period with reference to its respective unexposed group figure. This percent increase was also observed in susceptible population at 24, 48 and 72 h but then suddenly a percent decrease was prominent at 96 and 120 h of exposure with comparison to their respective control figures (Table III, Fig. 2a).

Free amino acids

The results showed that free amino acid contents in 4th instar larvae of susceptible and tolerant populations in unexposed groups were found elevated up to 96 h then started to decline. In exposed groups of both populations, an elevation was found till 48 h exposure which was followed by a decline in these contents. Moreover, the susceptible population possessed more free amino acids than the tolerant population. The level of free amino acid contents were significantly increased by 131, 135 and 39% in 4th instar larvae of susceptible population after exposure to phosphine for 24, 48 and 72 h, respectively with reference to unexposed group. Later on, these contents were significantly reduced when exposed to phosphine for 96 and 120 h with respect to its unexposed group values. On the other hand, the level of free amino acids in 4th instar larvae of tolerant population were significantly increased after each exposure period with respect to its unexposed group. It was also noticed that the percent increase in free amino acid contents in 4th instar larvae of both populations followed a decreasing trend as the exposure time proceeds (Table II, Fig. 1b).

The data regarding 6th instar larvae indicated that free amino acids contents increased in both populations throughout the period but in exposed groups the contents followed same trend up to 48 h of exposure then started to decrease at 72, 96 and 120 h of exposure. Both populations presented a significant percent increase in the concentration of free amino acids in 6th instar larvae at each exposure period with respect to unexposed group value (Table III, Fig. 2b).

Glucose contents

In unexposed groups of both populations, the glucose contents in 4th and 6th larval instars were consistently increased but after 96 h they started to decrease. The glucose contents first increased then started to decrease after 48 h exposure to phosphine in 4th instar larvae of both populations.

The percent increase (58, 56 and 21%) in glucose contents in 4th instar larvae of susceptible population after 24, 48 and 72 h exposure to sub-lethal dose of phosphine in comparison with the respective figures for control, respectively was followed by a percent decrease at 96 and 120 h. In case of tolerant population the significant increase (191, 175, 125 and 79%) in glucose contents for 24, 48, 72 and 96 h of phosphine exposure was shifted to a significant percent decrease at 120 hrs of exposure in comparison with the respective figures for control, respectively (Table II, Fig. 1c).

Conversely, these contents were significantly increased in 6th instar larvae of both populations after each exposure period. The glucose contents were significantly increased in tolerant population at each exposure period with respect to unexposed group value but in susceptible population glucose contents shoed percent decrease at 24 and 48 h but then they exhibited percent increase (21, 36 and 39%) at 72, 96 and 120 h respectively with comparison to unexposed group values (Table III, Fig. 2c).

Glycogen, trehalose, lipids and nucleic acids contents

The glycogen, lipid, nucleic acids and trehalose contents in unexposed and exposed groups of both larval instars of susceptible and tolerant populations were consistently decreased as the time period proceeded. It was evident that all the above mentioned parameters were significantly decreased after exposure to phosphine at each interval when compared to their respective unexposed groups. It was also revealed that percent decrease in these parameters was more prominent at later period of exposure (Tables II and III, Figs. 1d-1h and 2d-2h).

Trehalase and amylase activities

The trehalase and amylase activities in unexposed and exposed groups of 4th larval instars of both populations were slightly increased with the passage of time (Tables II and III, Figs. 1i and 1j). The activities of both enzymes in 6th larval instars of both populations were significantly increased with reference to their respective control at each exposure period (Tables II and III, Figs. 2i and 2j).

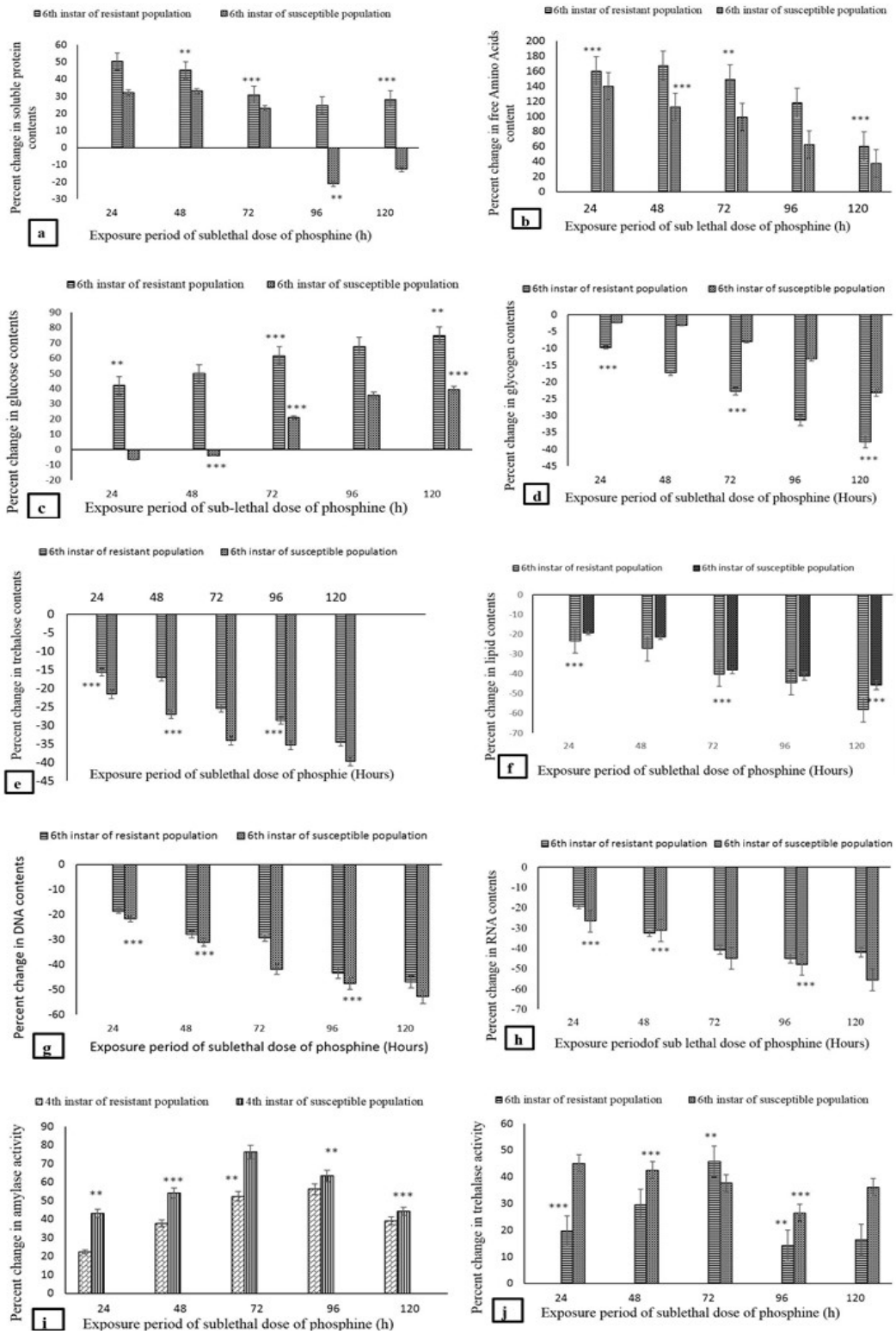

Fig. 2. Effect of phosphine administered at LC_{20} for different time periods on the percent changes in various biochemical parameters (a-j) with reference to control of 6th instar larvae of susceptible and tolerant populations of *T. granarium*. One way ANOVA $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

Table III. Effect of phosphine administered at LC_{20} for different time periods on carbohydrate metabolism and macromolecular concentration of 6th instar larvae of susceptible and tolerant populations of *T. granarium*.

Parameters	Exposure periods (h)	Control		Treated	
		Susc-eptible strain (n=20)	Tolerant strain (n=20)	Susceptible strain (n=20)	Tolerant strain (n=20)
Soluble protein (µg/mg)	24	17.70±0.4[a]	15.29±0.42[a]	23.4±0.45*	23.01±0.45*
	48	18.21±0.43	16.49±0.43	24.26±0.43*	23.99±0.43*
	72	18.75±0.43	18.49±0.43	23.09±0.60*	24.19±0.60*
	96	19.44±0.41	20.17±0.41	15.33±0.41*	25.14±0.41*
	120	17.16±0.53	20.23±0.53	15.01±0.42*	25.94±0.42*
Free amino acids (µg/mg)	24	41.45±0.48	43.23±0.48	99.57±0.44*	111.42±0.41*
	48	47.10±0.52	45.41±0.52	100.12±0.43*	121.42±0.43*
	72	50.79±0.41	45.92±0.41	101.21±0.48*	114.42±0.48*
	96	56.59±0.48	45.72±0.48	92.01±0.46*	99.77±0.46*
	120	58.88±0.40	45.04±0.40	81.11±0.63*	72.13±0.63*
Glucose (µg/mg)	24	52.72±0.42	74.17±0.42	49.31±0.42*	105.23±0.42*
	48	52.91±0.42	74.28±0.42	50.76±0.52*	111.23±0.52*
	72	51.56±0.44	73.88±0.44	62.42±0.40*	119.31±0.40*
	96	51.14±0.52	72.41±0.52	69.42±0.54*	121.34±0.54*
	120	49.76±0.51	71.15±0.51	69.32±0.44*	124.23±0.44*
Glycogen (µg/mg)	24	1.29±0.09	1.24±0.01	1.26±0.02*	1.12±0.02*
	48	1.27±0.01	1.22±0.01	1.23±0.02*	1.01±0.03*
	72	1.25±0.07	1.19±0.01	1.15±0.04*	0.92±0.04*
	96	1.22±0.07	1.15±0.01	1.06±0.04*	0.79±0.04*
	120	1.21±0.04	1.14±0.04	0.93±0.08*	0.71±0.08*
Lipids (µg/mg)	24	0.15±0.02	0.17±0.02	0.12±0.02	0.16±0.02
	48	0.15±0.03	0.17±0.03	0.12±0.02	0.12±0.02
	72	0.15±0.04	0.16±0.04	0.09±0.00*	0.11±0.00*
	96	0.14±0.01	0.15±0.01	0.08±0.01*	0.09±0.01*
	120	0.14±0.01	0.09±0.01	0.08±0.00*	0.06±0.00*
DNA (µg/mg)	24	0.05±0.05	0.04±0.05	0.04±0.00*	0.04±0.00*
	48	0.05±0.00	0.04±0.00	0.03±0.01*	0.03±0.01*
	72	0.04±0.01	0.04±0.01	0.03±0.01*	0.03±0.01*
	96	0.04±0.01	0.04±0.01	0.02±0.01*	0.02±0.01*
	120	0.04±0.01	0.03±0.01	0.02±0.01*	0.02±0.01*
RNA (µg/mg)	24	0.12±0.03	0.12±0.03	0.08±0.01*	0.12±0.01*
	48	0.12±0.01	0.12±0.01	0.0±0.01*	0.08±0.01*
	72	0.11±0.01	0.12±0.01	0.06±0.01*	0.07±0.01*
	96	0.10±0.00	0.11±0.00	0.05±0.01*	0.06±0.01*
	120	0.10±0.00	0.10±0.00	0.04±0.02*	0.06±0.02*
Trehalose (µg/mg)	24	11.21±0.03	11.21±0.03	8.79±0.03	9.45±0.03
	48	11.91±0.03	11.09±0.03	8.69±0.03	9.13±0.03
	72	12.71±0.03	11.01±0.03	8.11±0.03	8.21±0.03
	96	12.31±0.03	10.95±0.03	7.96±0.03	7.81±0.03
	120	11.81±0.03	10.81±0.03	7.12±0.03	7.07±0.03
Trehalase (IU/mg)	24	0.05±0.00	0.05±0.00	0.07±0.01*	0.06±0.01*
	48	0.05±0.01	0.05±0.01	0.07±0.01	0.07±0.01
	72	0.06±0.01	0.06±0.01	0.08±0.01	0.09±0.01
	96	0.07±0.01	0.06±0.01	0.09±0.01	0.07±0.01
	120	0.07±0.01	0.06±0.01	0.1±0.01	0.07±0.01
Amylase (IU/mg)	24	0.03±0.01	0.05±0.01	0.04±0.01	0.06±0.01
	48	0.04±0.01	0.05±0.01	0.05±0.01	0.07±0.01
	72	0.04±0.01	0.05±0.01	0.05±0.01	0.07±0.01
	96	0.04±0.01	0.06±0.01	0.06±0.01	0.07±0.01
	120	0.05±0.00	0.06±0.00	0.07±0.01*	0.09±0.01*

DISCUSSION

The literature reported that 0.05ppm Phosphine in stored grains and 0.01 ppm in other food was highly toxic and posses very severe concern to food safety (Kroeller, 1968). Therefore, the increase in fumigant concentration is not a recommended solution to overcome the problem of insect pest resistance development against fumigants because increase in fumigant concentration may also cause necrosis and eventual survival of the insect pest (Nakakita and Kuroda, 1986; Shakoori *et al*., 2016). The current study was focused on the role of exposure period in alteration of energy reserves and ultimate death of larval instars. The Haber (1924) rule was found valid in current investigation as the increased exposure period of phosphine caused almost 100% mortality at LC_{50} value and produced a significant impact on tested biochemical profile of *T. granarium*. Rauscher and Harbottle (1957) proposed that phosphine may affect more favorably in low concentration and prolonged time period. The results clearly showed 100% mortality by inducing alteration in all the tested profile of 4th and 6th larval instars of *T. granarium* in response to increased exposure period of sub lethal dose of phosphine from 24-120 h. This increased exposure time caused depletion in energy sources which ultimately leads to the death. Winks and Waterford (1986) suggested that phosphine found to be more effective for longer exposure time to control the red flour beetle, *Tribolium castaneum* (Herbst). Shakoori *et al*. (2016) also reported that exposure period is very critical factor for fumigation on working with adult beetles of *T. granarium*. Similarly, Daglish *et al*. (2002) found that exposure period is more important factor than phosphine concentration to control resistant populations of the rice weevil, *Sitophilus oryzae* (L.). Price and Mills (1988) and Sun (1946) elucidated that exposure time alone irrespective of phosphine concentration had a significant effect on pest management program. Our results revealed a positive relationship between exposure period of fumigant and depletion in energy reserve and ultimately increased mortality. Fumigant needs some time to produce its toxic impact on insect pest, so, time is indeed a very important factor for proper insect pest control.

Carbohydrates, proteins and lipids are the main constituents of the insect body and are responsible for various types of biochemical reactions. Carbohydrates are the major energy reservoirs that may involve in protein production and can be transformed into variety of lipids. Proteins have an important role in maintaining the structure and function of enzymes, formation of cuticle scleritization, induction of diapauses and synthesis of neural transmitters and pigments (Yazdani *et al*., 2013). Lipids are also basic biomolecules that are involved in formation of phospholipids, fatty acids and sterols. These are an integral constituent of insect cell walls and also vital in many other cellular processes (Chapman *et al*., 1998). The use of insecticides or fumigants over wide range of exposure can alter these important biochemical parameters (Etebari *et al*., 2005; Nath, 2002; Shekari *et al*., 2008; Yazdani *et al*., 2013). Hence, alterations in the metabolism of 4th and 6th larval instars of susceptible and tolerant populations of *T. granarium* can be expected under phosphine stress to fulfill energy demands over a wide range of exposure period.

The current study clearly presented an overall shift in glycogen and trehalose metabolism from 24-120 h of fumigation. The magnitude of changes was more prominent in 4th instar larvae than 6th instar larvae of *T. granarium*. Normally the glycogen and trehalose are found as main sugars in the hemolymph of insect body (Shukla *et al*., 2014) but the exposure of fumigant provides effective stimulus for conversion of glycogen and trehalose to glucose to provide energy source to cope the stress condition (Shakoori *et al*., 2018) and causes carbohydrate depletion (Shaurub and El-aziz, 2015). It was evident from current study that the rise in glucose and depletion in both glycogen and trehalose contents after exposure was due to this phenomenon. Nath (2002) supported the current findings by narrating that breakdown of homeostatic mechanism coupled with glycogenolysis to meet the required energy demands of the silkworms *Bombyx mori* (L.) under fenitrothion and ethion toxicity over exposure period of 1-7 d was the main cause of glycogen depletion. The decline in lipid content may be due to disturbance in lipid biosynthesis, lipid metabolism as well the utilization of lipids as an energy source to cope the stress (Shaurub and El-aziz, 2015) exerted by long term exposure of phosphine. This decline could be associated with hormonal dysfunction because the hormones which control lipid metabolism are disturbed and thus not secreted as reported by Yazdani *et al*. (2013). Mulye and Gordon (1993) reported that disturbance in cytosolic enzymes could cause deficiency of fatty acids for subsequent esterification and biosynthesis of complex lipids in an Eastern Spruce Budworm after exposure to juvenile hormone analogue (Fenoxycarb). Shakoori *et al*. (2016) reported a rise in glucose while decline in lipid and glycogen contents in adult beetles of *T. granarium* after phosphine exposure over wide range of period. Shakoori *et al*. (2018) on working with 4th and 6th larval instars of *T. granarium* after exposure to esfenvalerate and λ-Cyhalothrin also documented reduction in lipids, glycogen and trehalose contents while elevation in glucose contents.

The elevation in soluble protein and free amino acids

contents in untreated groups of both larval instars of both populations may be due to decreased level of GPT and GOT which results in reduced transamination. In start of exposure, increase of soluble and free amino acid contents in phosphine treated groups with reference to control group may be due to reduced activity of transaminases but after prolonged period of exposure the larval instars were found energy deficient so, the normal physiological and biochemical processes were altered and proteins were degraded to free amino acids which were entered to Kreb cycle as keto acids as suggested by Bizhannia *et al*. (2005) working on silkworm *B. mori* after treatment with juvenile hormone analogue. Nath *et al*. (1997) suggested that depletion in protein contents undergoes a physiological compensatory mechanism under stress conditions to keep continue the Krebs cycle and retained free amino acid content in hemolymph. Moreover, Sugumaran (2010) documented that volume of hemolymph decreased as a result of stress which ultimately reduced the protein contents. The decrease in RNA contents after prolonged period of insecticidal stress could result in reduced biosynthesis of protein reported by Shaurub and El-aziz (2015) and Shakoori *et al*. (2016) working on adult beetles of *T. granarium* after phosphine exposure over wide range of period, working with 4th and 6th instar larvae of *T. granarium* after exposure to esfenvalerate and λ-cyhalothrin reported elevated levels of free amino acids and soluble protein contents (Shakoori *et al*., 2018) in accordance with present investigations. Molting and juvenile hormones control the development of insects and these both hormones are regulated by various enzymes. Socha and Sehnal (1973) reported that synthesis of RNA is activated by molting hormone while duplication of DNA is induced by juvenile hormone. In current investigation the contents of DNA and RNA were decreased as phosphine exposure period was increased, thus suggesting that phosphine may inflict its toxicity by acting as insect growth regulators and targeting its molting and juvenile hormone (Shakoori *et al*., 2016).

Insect trehalase plays a vital role in growth and development, regulation of chitin biosynthesis, energy supply for flight and recovery from abiotic stress (Shukla *et al*., 2014) and catalyzes the conversion of trehalose into glucose. Similarly, amylase is a digestive enzyme that catalyzes the breakdown of 1-4-α-glucosidase bonds in polysaccharides and hence, the increased activity of amylase indicated that amylase plays an important role in conversion of glycogen to glucose and starch to maltose (Yazdani *et al*., 2013). The increased activities of amylase and trehalase in current study are evident from decreased contents of glycogen and trehalose. Nath (2002) reported elevated trehalase activities in silkworm, *B. mori* after exposure to sub lethal doses of ethion and fenitrothion. Moreover, Vyjayanthi and Subramanyam (2002) also investigated the increased activity of trehalase in *B. mori* after exposure to fenvalerate. There are different reports on the activity of amylases. Yazdani *et al*. (2013) reported decreased amylase activity in lesser mulberry pyralid *Glyphodes pyloalis* (Walker) after treatment with essential oils. Similarly, (Shekari *et al*., 2008) reported decreased amylase activity in elm leaf beetle; *Xanthogaleruca luteola* (Mull.) with treatment of methanolic extract of *Artemisia annua* (L.) and Vyjayanthi and Subramanyam (2002) also investigated the decreased activity of amylase in *B. mori* after exposure to fenvalerate. Shakoori *et al*. (2018) on working with 4th and 6th larval instars *T. granarium* after exposure to esfenvalerate and λ-Cyhalothrin reported reduced activity of amylase in contrast to present investigations. So, it is suggested that different insecticide posed different effect on this metabolic enzyme.

It is concluded that fumigant posed more pronounced effect on energy reserve over extended period of exposure so, due to alteration in biochemical parameters, larval system cannot withstand the stress and as a result 100% mortality was achieved. Therefore, it is necessary to fumigate the storage facilities for a longer period of time to cope the problem of insect pest development in storage commodities.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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