Archives of Insect Biochemistry and Physiology 66:23–31 (2007)

The Effects of Boric Acid-Induced Oxidative Stress on Antioxidant Enzymes and Survivorship in *Galleria mellonella*

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Larvae of the wax moth, *Galleria mellonella* (L.), were reared from first instar on a diet supplemented with 156, 620, 1,250, or 2,500 ppm boric acid (BA). The content of malondialdehyde (MDA, an oxidative stress indicator), and activities of the antioxidant enzymes [superoxide dismutase (SOD), catalase (CAT), glutathione S-transferase (GST), and glutathione peroxidase (GPx)] were determined in the fat body and hemolymph in the 7th instar larvae and newly emerged pupae. Relative to control larvae, MDA was significantly increased in larval hemolymph, larval and pupal fat body, but decreased in the pupal hemolymph. Insects reared on diets with 156- and 620-ppm BA doses yielded increased SOD activity but 1,250- and 2,500-ppm doses resulted in decreased SOD activity in larval hemolymph. SOD activity was significantly increased but CAT was decreased in the larval fat body. High dietary BA treatments led to significantly decreased GST activity. However, they increased GPx activity in larval hemolymph. Dietary BA also affected larval survival. The 1,250- and 2,500-ppm concentrations led to significantly increased larval and pupal mortality and prolonged development. In contrast, the lowest BA concentration increased longevity and shortened development. We infer that BA toxicity is related, at least in part, to oxidative stress management. Arch. Insect Biochem. Physiol. 66:23–31, 2007.

KEYWORDS: *Galleria mellonella*; boric acid; antioxidant enzymes; hemolymph; fat body; malondialdehyde; survivorship

INTRODUCTION

Pesticide chemicals produce reactive oxygen species (ROS), leading to oxidative stress and alterations in radical scavenging enzymes (Felton and Summers, 1995; Büyükgüzel, 2006). ROS cause lipid peroxidation, protein and enzyme oxidation, and glutathione (GSH) depletion in insects. Oxidative challenge is alleviated by antioxidant compounds, but more importantly by induction of antioxidant enzymes including superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione *S*-transferases (GST) (Ahmad, 1995). SOD, CAT, and GPx form a defense complex against ROS. SOD catalyzes the dismutation of superoxide radicals to H_2O_2 and oxygen and appears to be the main response to dietary prooxidant exposure. CAT reduces H_2O_2 to water and oxygen (Ahmad et al., 1991).

Beyond these general responses, proteolysis with a concomitant increase in protease and transaminase activities in hemolymph and fat body (Nath et al., 1997) and increased whole body protein content (Içen et al., 2005) are compensatory physiological mechanisms in response to oxidative stress. A significant increase in the protein carbonyl levels and a concominant increase in antioxidant enzyme activities were observed in *Spodoptera littoralis* larvae in response to oxidative stress from various dietary allelochemicals (Krishnan and Kodrik, 2006). Changes in activities of some antioxidant enzymes in hemolymph serve as bio-

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Received 31 October 2006; Accepted 14 February 2007

DOI: 10.1002/arch.20194
Published online in Wiley InterScience (www.interscience.wiley.com)

markers for oxidative stress in *G. mellonella* (Lozinskaya et al., 2004).

Chemical pest management programs rely upon applications of broad-spectrum pesticides that pose a threat to nontarget organisms and the environment (Charriere and Imdorf, 1997). These problems have focused attention onto less toxic natural insecticides and lesser-used compounds such as BA. BA, a nonvolatile, slow-acting inorganic insecticide, has long been used in urban pest management (Cochran, 1995). However, no comprehensive appreciation of boric acid (BA)–induced changes affecting biological fitness of insects has been established. Most BA toxicity studies have been directed toward biological fitness of adult urban pest insects (Zurek et al., 2003; Gore et al., 2004). It has been proposed that BA produces free radicals leading to oxidative damage in some tissues and increasing mortality (Habes et al., 2006). Elimination of various ingested allelochemicals is often associated with the production of oxidative radicals that damage insect tissues by oxidizing vital cell components in the midgut (Krishnan and Sehnal, 2006; Krishnan et al., 2006). The alkaline redox conditions in lepidopteran midguts (Terra et al., 1996) would facilitate the dissociation of BA resulting in the generation of ROS (Jolly, 1991). In the present study, we report on outcomes of experiments based on our hypothesis that dietary BA induces oxidative stress and influences anti-oxidant enzymes in the hemolymph and fat body of the wax moth, *Galleria mellonella*.

MATERIALS AND METHODS

Insects

Larvae of the greater wax moth, *Galleria mellonella* (L.) were used in all experiments. The insects were reared in 1,000-ml glass jars with an artificial diet (Bronskill, 1961), at 30 ± 1°C in constant darkness. The standard diet was composed of 420 g of wheat bran, 150 ml of filtered honey, 150 ml of glycerol, 20 g of ground old honeycomb, and 30 ml distilled water. Fifteen newly emerged adult females were placed in the jars and provided a piece of old honeycomb on the diet for egg deposition and feeding of newly hatched larvae. The methods used to prepare and dispense diets into containers and the methods used to obtain eggs and larvae and their placement onto diets have been described by Içen et al. (2005).

Experimental Designs

BA (Crystal form, 99%, H_3BO_3, Eti Mine Works General Management, Ankara, Turkey) was directly incorporated into diets at concentrations of 156, 620, 1,250, or 2,500 ppm. Using standard laboratory rearing conditions, two series of experiments were carried out to examine the effects of BA on the insect. In the first series of experiments, we determined the influence of dietary BA on lipid peroxidation levels and activities of antioxidant enzymes in hemolymph and fat body of last instar larvae (7th) and early pupae (1 day old). These stages were used in the biochemical analyses because our preliminary experiments demostrated that BA exerted its main effects mostly on the larval and pupal stages of the insects. In the second series, the effects of BA on survivorship, development, and adult longevity were determined.

Biochemical Assays

First instar larvae were reared through 7th instars on an artificial diet amended with given concentrations of BA. The larvae were transferred into another jar lined with a filter paper for pupation. Last-instar larvae (upon reaching 7th instars, 100–150 mg) and newly emerged pupae (90–100 mg) were used for determining the content of the lipid peroxidation product MDA and antioxidant enzymes activities. All larvae and pupae were the same chronological age. The larvae and pupae were chilled on ice for 5 min and surface sterilized in 95% ethanol. Larval hemolymph was collected into cold Eppendorf tubes by amputating the second pair of prolegs. Pupal hemolymph was collected into a cold tube by puncturing heads of pupa with

a fine syringe needle (Hyršl and Šimek, 2005). Fat body was dissected from larvae and pupae into a cold homogenisation buffer (w/v 1.15% KCL, 25 mM K_2HPO_4, 5 mM ethylen-diaminetetraacetic acid EDTA, 2 mM phenylmethylsulphonil fluoride PMSF, 2 mM dithiotreitol DDT, pH 7.0). A few crystals of phenylthiourea (PTU) were added to each sample to prevent melanization. The samples were frozen at –25°C until use.

Sample Preparation and Determination of Malondialdehyde (MDA) Content and Antioxidant Enzymes Activities

Extracts of hemolymph and fat body were prepared at 4°C by an ultrasonic homogenizer (Bandelin Sonoplus, HD2070, Berlin, Germany) at 50 W, 40–50 s in homogenisation buffer and subsequent centrifugation at 10,000*g* for 10 min. The resulting cell-free extracts were collected for biochemical analysis. Assays were replicated four times each with five larvae or pupae. Protein concentrations were determined according to Lowry et al. (1951) by using bovine serum albumin (BSA) as a quantitative standard. All chemicals were analytical grade and were obtained from Sigma-Aldrich (St. Louis, MO). A dual beam spectrophotometer (Shimadzu 1700, UV/VIS Spectrophotometer, Kyoto, Japan) was used for all absorbance measurements.

Malondialdehyde (MDA) contents were assayed according to Jain and Levine, (1995). MDA reacts with thiobarbituric acid (TBA) to form a colored complex. MDA contents as an indicator of lipid peroxidation were determined after incubation at 95°C with TBA (1% w/v). Absorbances were measured at 532 nm to determine MDA content. MDA content were expressed as nmol/mg protein by using 1.56×10^5 $M^{-1}cm^{-1}$ for extinction coefficient.

Total SOD (EC 1.15.1.1) activity was determined according to Marklund and Marklund (1974) assaying the autooxidation and illumination of pyrogallol at 440 nm for 3 min. One unit total SOD activity was calculated as the amount of protein causing 50% inhibition of pyrogallol autooxidation. The total SOD activity was expressed as units per miligram of protein ($U.mg^{-1}$).

CAT (EC1.11.1.6) activity was measured according to Aebi (1984) assaying the hydrolyzation of H_2O_2 and decreasing absorbance at 240 nm over a 3 min at 25°C. CAT activity was expressed as milimoles of H_2O_2 reduced per minute per milligram of protein, using $\varepsilon_{240} = 0.0394$ $mM^{-1}cm^{-1}$.

GST (EC 2.5.1.18) activity was assayed by measuring the formation of the GSH and 1-chloro-2,4-dinitrobenzene (CDNB) conjugate (Habig et al., 1974). The increase in absorbance was recorded at 340 nm for for 3 min. The specific activity of GST was expressed as nmol GSH-CDNB conjugate formed/min/mg protein using an extinction coefficient 9.6 $mM^{-1}cm^{-1}$.

GPx (EC1.11.1.9) activity was measured with H_2O_2 as substrate according to Paglia and Valentine (1987). This reaction was monitored indirectly as the oxidation rate of NADPH at 340 nm for 3 min. Enzyme activity was expressed as nmol of NADPH consumed per minute per milligram of protein, using an extinction coefficient 6220 $M^{-1}cm^{-1}$.

All assays were corrected for nonenzymatic reactions using corresponding substrate in phosphate buffer (50 mM, pH 7.0).

Survivorship, Development, and Adult Longevity

Developmental time from first instars to 7th-instar, pupal, and adult stage, survivorship in these stages, and adult longevity were recorded as biological fitness indicators. The 7th-instar larvae were transferred into another jar lined with a filter paper for pupation and then adult emergence. Number of pupae and adults were recorded, and their developmental times were calculated for each replication. Each experiment including four BA concentrations and one control was replicated four times with 20 larvae. Newly emerged females adults from the experiments were transferred into 30-ml plastic cups (35 × 55 mm, OrLab Ltd., Ankara, Turkey) covered with screen lid. To determine average adult longevity, the number of dead adults in each of the treatment groups was recorded every day till all adults died. Adult longevity experiments were conducted at 25°C. Experiments were replicated four times with 10 adults per replication.

Statistical Analysis

Data on the MDA content, antioxidant enzymes activities, and development and longevity, were evaluated by one-way analysis of the variance (ANOVA). To determine significant differences between means, a least significant difference (LSD) test (PROC GLM, SAS Institute, 1989) was used. Data on survivorship were compared by a chi-squared test (Snedecor and Cochran, 1989). When the F and χ^2 estimate exceeded the probability of 0.05, the differences were considered significant.

RESULTS

MDA Content and Antioxidant Enzymes Activities

The influence of dietary BA on MDA content is displayed in Figure 1. Dietary BA led to increased MDA content in larval hemolymph and fat body in a dose-dependent manner (Fig. 1a). MDA content increased with dietary BA concentrations in pupal fat body but not in pupal hemolymph (Fig. 1b).

Dietary BA treatments led to altered SOD activities (Fig. 2). The diets supplemented with 156 and 620 ppm BA led to increased SOD activity but 1,250- and 2,500-ppm doses resulted in decreased SOD activities in larval hemolymph. Dietary BA led to increased SOD activities in larval fat body, although these increases were not directly linked to dietary BA concentrations (Fig. 2a). Dietary BA treatments led to increased SOD activity in pupal fat body, but not hemolymph (Fig. 2b).

Our results with respect to CAT activity are displayed in Figure 3. Dietary BA resulted in significantly decreased CAT activity in larval hemolymph and fat body (Fig. 3a). Dietary BA led to increased CAT activities in pupal hemolymph and fat body (Fig. 3b). Diet with 620 ppm BA resulted in significantly increased CAT activity by about 20-fold in pupal hemolymph and by about 4-fold in pupal fat body.

Dietary BA did not exert a pattern of influence on larval or pupal GST activities (Fig. 4). On the other hand, higher dietary BA concentrations let to slight increases in larval hemolymph and (for

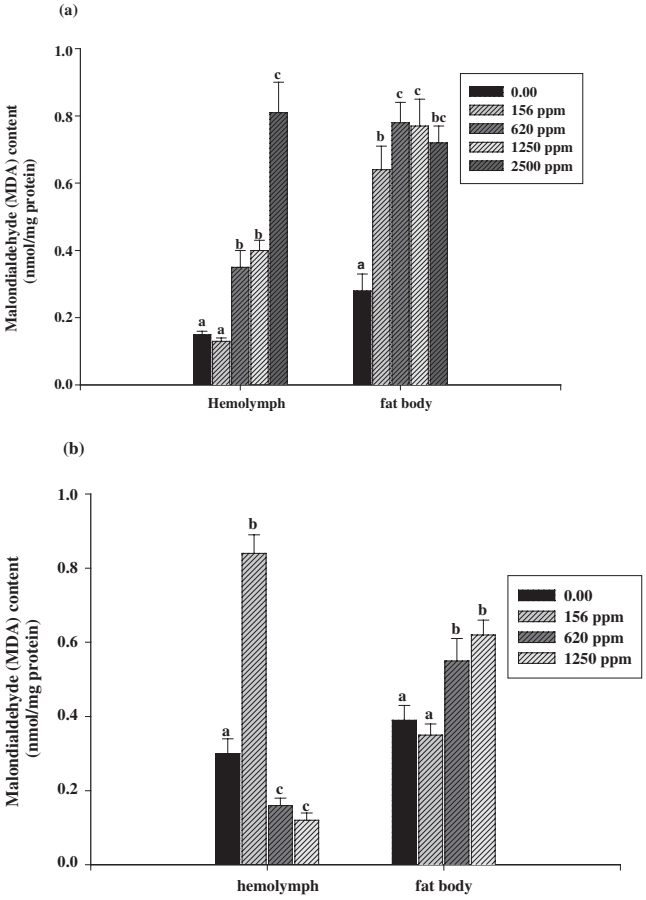

Fig. 1. Effects of boric acid on MDA content in hemolymph and fat body of *G. mellonella* last instars (**a**) and pupae (**b**). Bars represent the means (± S.E.) of four replicates. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

620-ppm treatments) fat body GPx activities (Fig. 5a). The diets amended with 156 ppm BA led to increased GPx activities in larval (by 3-fold) and pupal (by 2-fold) fat body (Fig. 5b).

Survivorship, Development, and Adult Longevity

The influence of dietary BA on survivorship, development, and longevity of larvae reared on diets supplemented with BA are shown in Table 1. Dietary BA led to significantly reduced survival of 7th instar, and the reduced survival was registered in a dose-dependent manner, from very high larval survival in the absence of BA to less than 10% sur-

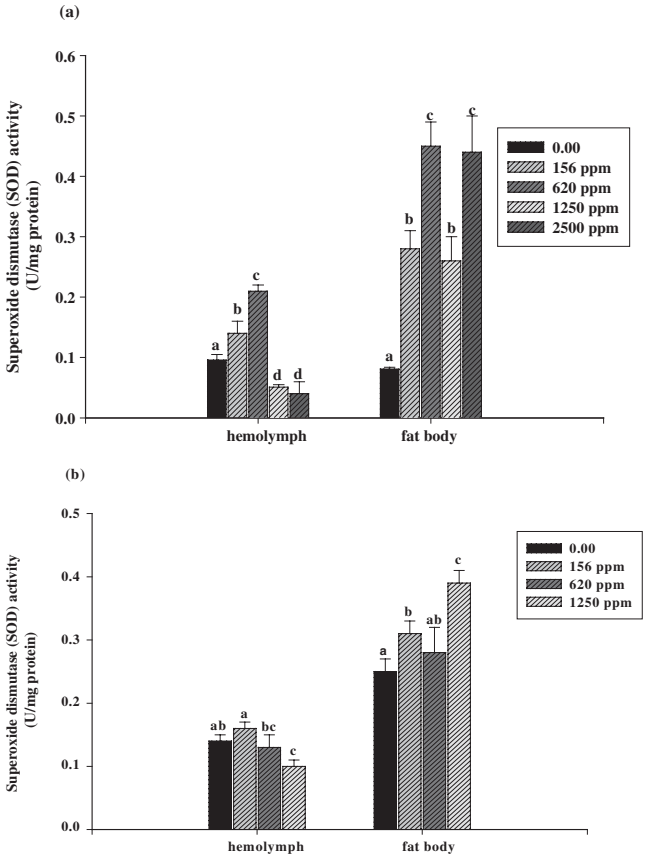

Fig. 2. Effects of boric acid on SOD activity in hemolymph and fat body of *G. mellonella* last instars (**a**) and pupae (**b**). Bars represent the means (± S.E.) of four replicates. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

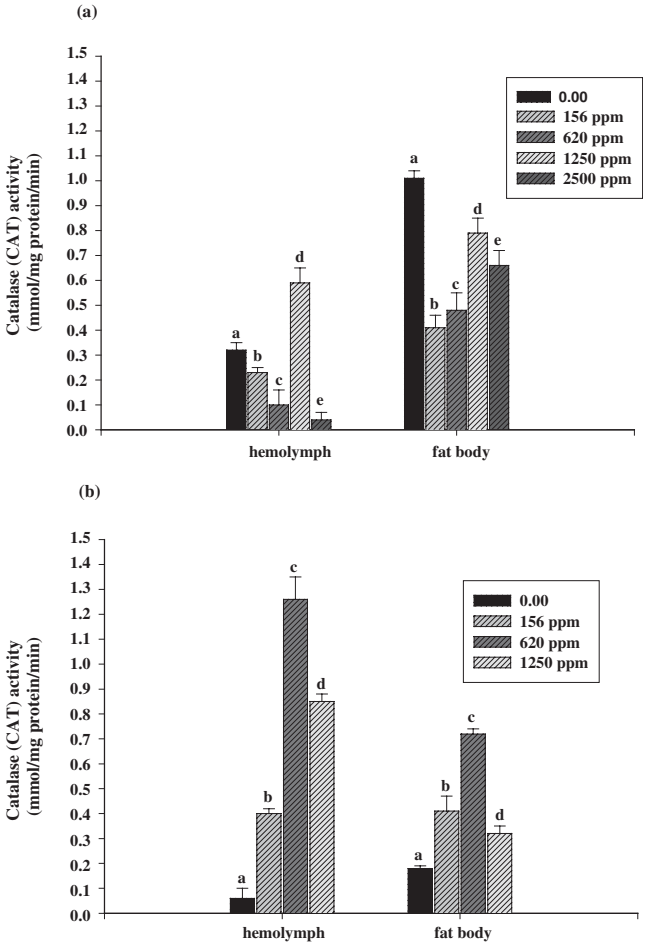

Fig. 3. Effects of boric acid on CAT ativity in hemolymph and fat body of *G. mellonella* last instars (**a**) and pupae (**b**) . Bars represent the means (± S.E.) of four replicates. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

vival in the presence of 2,500 ppm BA. BA treatments similarly reduced survival to pupal and adult stages. Coincident with these negative effects on survival, developmental times to 7th instars, pupa, and adults were prolonged in a dose-related manner. Adult longevity was reduced in a dose-dependent way.

DISCUSSION

The data reported in this study support our hypothesis that dietary BA treatments influence the biological fitness of wax moth larvae. The mechanism appears to be related to the effects of BA-induced oxidative stress on the anti-oxidant systems as shown by the influence of BA on MDA contents, changes in anti-oxidative enzymes, and declines in important life-table parameters.

Lipid peroxidation levels, as recorded by MDA concentrations, vary across tissues, developmental stages, and BA concentrations. Based on studies of insect species exposed to pro-oxidant environmental pullants (Ahmad, 1995; Cervera et al., 2003), we speculate that BA-induced elevated MDA content in the hemolymph and fat body followed from ROS production and reduced non-enzymatic protection from pro-oxidants during exposure to BA. Increased activities of antioxidant enzymes in the fat body and hemolymph are consistent with increased rates of adaptive metabolic responses to

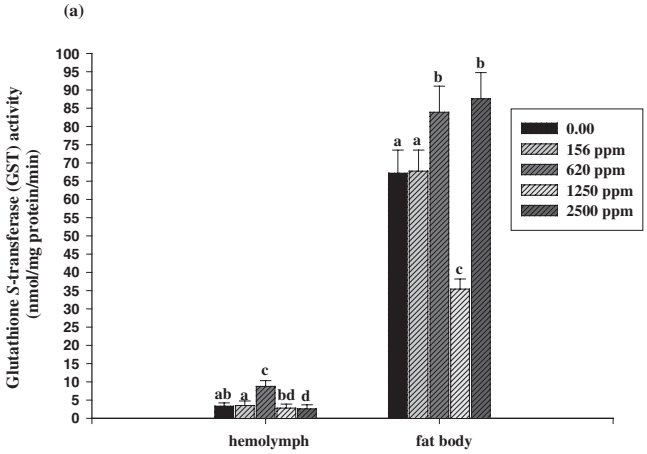

Fig. 4. Effects of boric acid on GST activity in hemolymph and fat body of *G. mellonella* last instars (**a**) and pupae (**b**). Bars represent the means (± S.E.) of four replicates. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

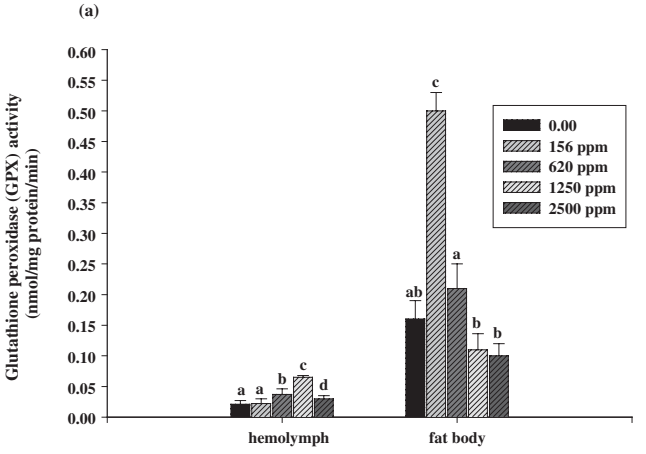

Fig. 5. Effects of boric acid on GPX activity in hemolymph and fat body of *G. mellonella* last instars (**a**) and pupae (**b**). Bars represent the means (± S.E.) of four replicates. Means followed by the same letter are not significantly different ($P > 0.05$, LSD test).

elevated lipid peroxidation. The damaging mechanism may be a quantative issue in which the amounts of pro-oxidant in the diets appear to overtake the protective mechanisms, leading to long-term oxidative stress.

SOD and CAT activities were elevated in the hemolymph and fat body of *G. mellonella* pupae exposed to the lowest BA concentration. Pritsos et al. (1990) demonstrated that SOD and CAT activities were significantly increased in some lepi-

TABLE 1. Effects of Boric Acid on Survivorship, Development and Adult Longevity of *G. mellonella* Reared on Artificial Diet Supplemented With the Indicated Concentrations of Boric Acid*

Boric acid concentration (ppm)	Survival to seventh instar (%)	Time to seventh instar (d) (Mean ± SD)	Survival to pupal stage (%)	Time to pupal stage (d) (Mean ± SD)	Survival to adult stage (%)	Time to adult stage (d) (Mean ± SD)	Adult longevity (d) (Mean ± SD)
0.00	98.7 a	22.4 ± 0.73 a	41.2 a	27.0 ± 0.63 ab	27.5 a	34.6 ± 0.48 a	8.4 ± 0.27 a
156	46.2 b	22.1 ± 0.53 a	45.0 a	26.2 ± 0.46 b	40.0 a	31.5 ± 0.85 b	11.8 ± 0.31 b
620	38.7 b	24.8 ± 0.71 b	25.0 b	28.2 ± 0.69 ac	23.7 ab	34.2 ± 0.56 a	5.6 ± 0.20 cd
1250	30.0 bc	25.8 ± 0.67 b	25.0 b	29.6 ± 0.56 c	12.5 b	34.3 ± 0.52 a	6.0 ± 0.31 c
2500	7.5 c	27.5 ± 0.76 b	5.0 c	37.0 ± 0.71 d	5.0 c	43.0 ± 0.55 c	5.0 ± 0.39 d

*Survival is represented as proportions of living insects. Developmental times are recorded in days (d). The reported data are means of four replicates with 20 larvae per replicate. Values followed by the same letter are not significantly different from each other, $P > 0.05$.

dopteran insects following exposure to generators of superoxide anions. CAT activity was also decreased in hemolymph and fat body of larvae reared on diets with the lowest BA concentration. SOD activities were increased with decreased CAT activity in larval hemolymph and fat body. The apparent inverse relationship between SOD and CAT activities aligns with similar observations by Felton and Summers, (1995).

Increased GST activities in correlation to elevated MDA content in hemolymph and fat body suggests this enzyme may have a protective role against BA-induced oxidative stress. GST plays a vital role in prevention of oxidative damage by conjugating reactive species and by detoxifying lipid peroxidation products (Singh et al., 2001). GSTs are thus a major family of detoxification enzymes confering insecticide resistance in insects. Induction of GST activity has been reported in many insects following severe toxicity of insecticides (Yu, 2004). This is consistent with our results showing high GST activities are found in the larval and pupal fat body exposed to the highest concentration of BA. Increased activity of GST and GPx in larval and pupal fat body of *G. mellonella* could represent a way of preparing the insect for adaptive metabolic response to the elevation of lipid peroxidation leading to BA toxicity (Ahmad et al., 1989). The increasing GPx activities attending decreasing GST activities at some BA concentrations suggests to us that rearrangement of separate activities of individual GST isoforms (GSTpx) takes place during BA stress. This has been suggested for another lepidopteran (Krishnan and Sehnal, 2006) under various environmental stressors.

Our study showed that survivorship and developmental time of wax moth larvae were adversely affected by BA doses. However, the lowest concentration of dietary BA resulted in extended adult longevity, and shortened developmental time to adulthood and slightly increased survivorship of pupae and adults. In urban pests, BA at high concentrations resulted in toxic or nutritionally unsuitable diets, which in turn led to decreases in various life-table parameters (Zurek et al., 2003; Gore et al., 2004). Our results showed that larvae reared on the diets supplemented with 2,500 ppm BA could produce only about 5% adult yield. However, our observation indicates that low dietary BA concentrations led to increased larval feeding rates. We surmise that the increased toxicity of dietary BA at sublethal concentrations may be due to increased ingestion (unpublished observations). Habes et al. (2006) reported that diets amended with increasing doses of BA damaged epithelial cells and impaired antioxidant defense in midgut of *Blatella germenica* causing increased mortality. A similar mechanism has been suggested for various pest insects exposed to BA in different baits (Cochran, 1995).

Our data show that antioxidant enzyme activities were not consistently related to increasing BA concentrations. We recorded the same trend in survival and development. A similar inconsistency in activities of detoxification enzymes in some lepidopteran moths was obtained by various dietary supplements (Hemming and Lindroth 2000). Büyükgüzel (2001) demonstrated that sublethal effects on life parametes of a dietary nonnutritive supplement may depend on altered larval feeding rates due to its interaction with dietary nutrients. It is thus reasonable to suggest that each dietary concentration of BA may exert different effects on the biological and biochemical response of insects. In agreement with this suggestion, our data show extended longevity and shortened development due to sublethal doses of BA in *G. mellonella*. We infer that low levels of BA may have stimulated the life parameters by induction of hemolymph and fat body antioxidant enzymes. Survival is concurrently increased with increasing detoxication enzyme activities in response to insecticide stress in adults of another lepidopteran insect (Adamski et al., 2003). These findings align with the work of Büyükgüzel (2006) who reported that increased SOD activities are related to increased survivorship, fecundity, and extented longevity of other insect groups under mild insecticidal toxicity.

ACKNOWLEDGMENTS

We are grateful to Dr. Natraj Krishnan (Czech Academy of Sciences, Institute of Entomology, De-

partment of Physiology, Ceske Budejovice, Czech Republic) for reading and providing useful comments on a draft of this report.

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