Effects of Ultrasound-Stress on Antioxidant Enzyme Activities of *Helicoverpa Armigera* (Hübner) (Lepidoptera: Noctuidae)[1]

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ABSTRACT Noctuid moths with tympanal organs perform a series of evasive maneuvers when exposed to bat-like ultrasounds. In this paper, we tested the hypothesis that certain ultrasound frequencies are environmental stress factors that have physiological effects on noctuid moths. The effects of ultrasound produced from a commercial device on the antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX), were investigated in the adults, pupae, and larvae of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). Our results showed that the effects of ultrasound-stress on antioxidant enzymes depended on the developmental stages of *H. armigera* and the duration of exposure. A significant ($P < 0.01$) increase in POX activity in adult and larval *H. armigera* was observed 40 minutes after ultrasound exposure. The results indicated that ultrasound stress has the potential to alter the antioxidant enzyme system in *H. armigera*.

KEY WORDS superoxide dismutase, catalase, peroxidase

Nocturnal moths have developed capabilities to deal with vertebrate natural enemies, such as insectivorous bats (Chiroptera), in order to ensure their survival. For instance, noctuid moths (Lepidoptera: Noctuidae) have bilateral tympanal organs on the metathoracic segment that are capable of detecting the echolocation calls of insectivorous bats (Zha et al. 2009). The tympana of noctuid moths are sensitive to ultrasound in the range of 10 kHz to 100 kHz, but they are best at detecting frequencies between 20 kHz and 50 kHz (Waters 2003). Noctuid moths show a series of maneuvers, including unpredictable loops, dives, rolls, and turns, to avoid capture by bats (Spangler 1988). It has been reported that mating behaviors and reproduction of moths are affected by ultrasound produced by hunting bats (Acharya & McNeil 1998). For example, ultrasound significantly reduced the oviposition of *Trichoplasia ni* (Hübner) (Lepidoptera: Noctuidae) in lettuce and broccoli plots (Payne & Shorey 1968).

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The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a major pest threatening cotton and food production in China (Ming et al. 2006). It has been reported that *H. armigera* of both sexes are capable of emitting ultrasonic signals while flying (Xue et al. 1996). They also can detect ultrasound, especially in the frequencies between 15 kHz and 30 kHz (Fullard et al. 2007). It has been reported that ultrasound-stress could modulate the cholinergic system in *H. armigera* (Zha et al. 2008). In addition, Agee & Webb (1969) found that ultrasound (20–40 kHz, 70–90 dB) can reduce the fecundity of *H. armigera*. Therefore, it appears that certain frequencies of ultrasound may be regarded as an environmental stress factor for *H. armigera*, and that ultrasound has a significant negative impact on insect physiology and ecology.

Oxidative stress is a common stress action for aerobic organisms, which often results from the production of reactive oxygen species (ROS) (Barbehenn et al. 2001). ROS entities contain one or more reactive oxygen atoms. For example, the superoxide radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH) may all cause oxidative damage to lipids, proteins, carbohydrates, and nucleic acids (Damien et al. 2004, Li et al. 2005). Insects, similar to other eukaryotes, possess a suite of antioxidant enzyme systems that scavenge ROS (Aucoin et al. 1991, Ahmad 1992, Weirich et al. 2001). The antioxidant system comprises three types of primary antioxidant enzymes; they are superoxide dismutase (SOD), catalase (CAT), and peroxidase (POX) (Manikandan & Devi 2005). SOD converts O_2^- to molecular O_2 and H_2O_2 at rates limited only by diffusion, and the accumulation of H_2O_2 is prevented in the cell by CAT and POX (Fridovich 1978, Barbehenn et al. 2001, Wang et al. 2001, Mittapalli et al. 2007). It has been demonstrated that the activities of SOD, CAT, and POX are influenced both in plant (Wang et al. 2003) and in animal species (Samson et al. 2005) by sound, but less is known about the activity of insect antioxidant enzymes in response to sound stress.

We studied the changes in different developmental stages of *H. armigera* after ultrasound exposure with different stimulation durations. We also studied whether ultrasound can induce alterations in these free radical scavenging enzymes. Using seven different stimulation durations of ultrasound exposure, we studied whether the observed changes are stimulation duration dependent and whether body adaptation to ultrasound occurs earlier or later. To understand whether the effects of the ultrasound are predominant in any developmental stage, three developmental stages of *H. armigera* were studied.

Materials and Methods

Insects. Larvae of *Helicoverpa armigera* were reared on artificial diet according to Wu & Gong (1997) at 27±1°C, 65±5% RH, and an L14:D10 photoperiod. Neonates were reared in glass dishes (30 cm diam. × 10 cm high). Larvae were transferred as second instars to glass tubes (3 cm diam. × 11.8 cm high) where they were reared until fully grown. Larvae were selected by sex according to the color of the body surface (males are red-brown, females are green). Pupae were segregated by sex, placed into separate test tubes (3 cm diam. × 11.8 cm high), and incubated at 27±1°C. Male pupae have a gonopore on

the 9th abdominal segment, whereas female pupae have a genital pore on the 8th abdominal segment. Adult moths were held in 100-ml clear plastic containers and fed a 10% honey solution on cotton.

Ultrasound experiment. A commercial ultrasonic device, LHC20 (Lihui, Inc., Wuhan, China), was used in our experiments. This piece of equipment has been used for arthropod pest management. The LHC20 generated peak frequencies at 33 and 69 kHz. It produced a 97 dB sound pressure level (SPL) at a distance of 50 cm from the source. The waveform plot showed that the ultrasound pulse width was 0.02 sec. SPL distributions within a cage were slightly different between 90 and 97 dB (Zha et al. 2008).

Moths (0–24 hours old), pupae (24–48 hours old), and larvae (third-fourth instars) were separated into seven groups containing ten females each. Males were not used in this experiment. Each group was exposed to one of the six ultrasonic exposure (20–50 kHz and 70–80 dB) durations from 10 to 60 min in 10 min intervals. The control group was not exposed to ultrasound. Each group represented a replicate and each exposure duration was replicated three times. All of the sample collections were done between 09:00 and 10:00 a.m. in order to avoid induced variation by circadian rhythm.

Enzyme extraction. The enzyme extraction was done using a kit (Cat. No. A045) purchased from Nanjing Jiancheng Bioengineering Institute [Nanjing, People's Republic (P. R.) of China]. Five insects were selected from each group for the extraction process. Samples were homogenized at 4°C in a glass homogenizer with insect saline (150 mM NaCl, 3 mM KCl, 4.9 mM $MgCl_2$, 1.5 mM NaH_2PO_4, 0.6 mM $NaHCO_3$, pH 7.4) (Yack 1993) in a proportion of 0.1 g of body weight to 1 ml of saline. The homogenate was centrifuged at 10,000 g for 20 min at 4°C, and the supernatant was stored on ice for determination of enzyme activity. The protein concentration of all samples was determined according to Bradford (1976) with bovine serum albumin as a standard.

Activity assay. The activities of SOD, CAT, and POX were determined according to the manufacturer's protocols from commercial kits (Nanjing Jiancheng Bioengineering Institute, Jiangsu, P. R. China), with some modifications (Meng et al. 2009). SOD activity was measured at 550 nm in the spectrophotometer with xanthine and xanthine oxidase systems. One unit of SOD activity was defined as the amount of enzyme required to cause 50% inhibition of the xanthine and xanthine oxidase system reaction in 1 ml enzyme extraction of 1 mg protein. SOD activity was expressed as U mg^{-1} protein. CAT activity was measured at 240 nm in the spectrophotometer due to H_2O_2 decomposition. One unit of CAT activity was defined as the amount that decomposes H_2O_2 per second per g protein. CAT activity was expressed as U mg^{-1} protein. POX activity was measured at 420 nm in the spectrophotometer by catalyzing the oxidation in the presence of H_2O_2 of a substrate. One unit of POX activity was defined as the amount that catalyses 1 mg substrate per minute per mg protein. POX activity was expressed as U mg^{-1} protein.

Data analysis. All data were analyzed by one-way analysis of variance (ANOVA) using GraphPad Prism (version 4.0 for Mac, GraphPad Software, San Diego, CA). When there were significant differences, Tukey's Honestly Significant Difference (HSD) test ($P < 0.05$) was used to make multiple comparisons and separate the means.

Results and Discussion

SOD activity. After different exposure times, the SOD activities in adults, larvae, and pupae did not change significantly ($P > 0.05$) compared to the control (Table 1).

CAT activity. In addition, the analysis of data showed that the CAT activities for different developmental stages of *H. armigera* did not change significantly ($P > 0.05$) for different exposure times to ultrasound (Table 2). The activities of CAT were between 78.6% and 111.1% of the control value in adults, and between 71% and 117.4% of the control value in pupae.

POX activity. For both adults and larvae, ultrasound had a significant effect ($P < 0.01$) on POX activity (Table 3). Ultrasound exposure of 40 min caused a 2.5-fold elevation in the enzyme activity of adults. For larvae, the activity of POX was 1.7-, 1.8-, 4.3-, 2.3-, 3.8-, and 1.8-fold of the control for exposure periods of 10, 20, 30, 40, 50, and 60 min, respectively (Table 3). The activity of POX in pupae did not change significantly after ultrasound exposure, and they were between 80.1% and 124.8% of the control value.

Certain frequencies of ultrasound can be regarded as noise stress to adults of *H. armigera*. For guinea pigs, levels of ROS in the cochlea increase following noise exposure (Yamane et al. 1995, Karlidağ et al. 2002). The increase of ROS could cause cytotoxic effects to the cells through a variety of mechanisms, including lipid and protein oxidation, damaging DNA strands, disrupting protein synthesis, or inactivating important cellular enzymes (Halliwell 1992, Ohinata et al. 2000, Van Campen et al. 2002).

This study shows that the POX activities change at varying degrees after ultrasound exposure of different durations. These irregular changes are similar to those in adult *H. armigera* after light exposure of different stimulation

Table 1. Effects of ultrasound exposure time on superoxide dismutase (SOD) activities for different developmental stages of *Helicoverpa armigera*.

	Superoxide dismutase activity (U/mg protein)[a] in *Helicoverpa armigera*		
Time (min)	Adults	Pupae	Larvae
Control	65.7 ± 14.3	73.2 ± 2.7	48.2 ± 2.8
10	59.2 ± 10.4	87.9 ± 5.5	36.7 ± 1.5
20	66.5 ± 12.3	81.4 ± 4.4	43.3 ± 3.2
30	57.7 ± 9.8	77.0 ± 6.1	40.6 ± 3.7
40	73.3 ± 17.3	82.7 ± 3.6	45.8 ± 3.4
50	63.1 ± 14.1	86.0 ± 2.2	43.9 ± 3.3
60	69.3 ± 14.8	86.7 ± 1.6	39.7 ± 1.7
F	0.17	1.80	1.82
df	20	20	20
P	0.982	0.171	0.166

[a]Values are expressed as Mean±SE. Each mean is based on n = 3 replications.

Table 2. Effects of ultrasound exposure time on catalase (CAT) activities for different developmental stages of *Helicoverpa armigera*.

	Catalase activity (U/mg protein) in *Helicoverpa armigera*[a]		
Time (min)	Adults	Pupae	Larvae
Control	2.6 ± 0.3	7.4 ± 1.6	3.7 ± 0.3
10	2.9 ± 0.3	5.9 ± 1.4	3.0 ± 0.1
20	2.7 ± 0.2	5.2 ± 1.1	3.1 ± 0.1
30	2.1 ± 0.3	7.8 ± 1.4	3.4 ± 0.1
40	2.6 ± 0.3	6.2 ± 0.9	3.7 ± 0.1
50	2.9 ± 0.2	8.6 ± 1.1	3.3 ± 0.2
60	2.6 ± 0.3	7.6 ± 1.0	3.2 ± 0.2
F	1.07	0.96	2.40
df	20	20	20
P	0.426	0.485	0.083

[a]Values are expressed as Mean±SE. Each mean is based on n = 3 replications.

durations (Jing, unpublished data). In chinchillas, the repeated exposure to noise causes a shift in the auditory threshold (Bohne & Harding 2000), and this may result in a fluctuation of the SOD level (Samson et al. 2005). We hypothesize that the fluctuation of the three antioxidant enzyme activities in adult *H. armigera* may also result from these same reasons.

Table 3. Effects of ultrasound exposure time on peroxidase (POX) activities for different developmental stages of *Helicoverpa armigera*.

	Peroxidase activity (U/mg protein) in *Helicoverpa armigera*[a]		
Time (min)	Adults	Pupae	Larvae
Control	2.8 ± 0.4bc[b]	2.1 ± 0.5	3.0 ± 0.3c
10	2.1 ± 0.5c	1.8 ± 0.5	5.1 ± 0.4bc
20	4.7 ± 0.3abc	2.6 ± 0.5	5.5 ± 0.2b
30	2.2 ± 0.2c	1.7 ± 0.1	12.9 ± 0.6a
40	7.0 ± 1.0a	2.3 ± 0.3	7.0 ± 0.5b
50	5.0 ± 0.7ab	2.2 ± 0.3	11.3 ± 0.7a
60	3.5 ± 0.5bc	1.8 ± 0.2	5.3 ± 0.6bc
F	10.53	0.77	50.37
df	20	20	20
P	0.0002	0.603	<0.0001

[a]Values are expressed as Mean±SE. Each mean is based on n = 3 replications.
[b]Means within a column followed by the same letters are not significantly different at $\alpha = 0.05$ (Tukey's HSD test).

It was reported that POX may be associated with the scavenging of H_2O_2, and an increase in POX activity is related to increase in stress tolerance (Clavaron-Mathews et al. 1997). In our experiment, the POX activity increased significantly compared to the control after 40 min of ultrasound exposure, indicating that oxidative damage occurred in adults when exposed to ultrasound stress. Ultrasound stress to POX was severe in *H. armigera* adults at longer exposure time. POX can metabolize lipid peroxides (Claravon-Mathews et al. 1997), which may potentially be very harmful in insects (Zhang et al. 2011). A significant increase in POX activity in response to ultrasound at longer exposure time and a simultaneous stable in CAT activity suggested that POX may have a more important role in scavenging H_2O_2 than CAT at longer exposure times. The reason may be that CAT removes H_2O_2 inefficiently at low cellular concentrations, and POX breaks down to safe levels (Ahmad & Pardini 2008). To the best of our knowledge, this is the first report to observe with the activities of SOD, CAT, and POX in moths after ultrasound exposure.

For pupae and larvae, ultrasound can also generate different ROS-like types of radiation and microwave radiation (Sies 1997). Our experimental data provide the evidence for this. It appears that the increase in SOD activity may have actually occurred during ultrasound exposure in pupae, although this was not statistically significant due to high variance. Although the activities of SOD and CAT did not change significantly ($P > 0.05$) after ultrasound exposure, increases of POX activity in larvae supported the hypothesis that increased oxidative stress leads to an up-regulation of antioxidant enzymes in lepidopteran larvae (Krishnan & Kodrik 2006). These results indicate that oxidative damage occurred in the pupae and larvae of *H. armigera* when exposed to ultrasound-stress. These results may explain why the pupation rate and the eclosion rate in the presence of ultrasound are lower than those in the absence of ultrasound (Zha et al., unpublished data).

In this study, exposure to ultrasound after different durations produced a remarkable increase in the POX activities of adult and larvae of *H. armigera*, which results in the formation of increased production of H_2O_2. The second line of defense is the enzymes that convert H_2O_2 into water and molecular oxygen, with the two enzymes involved being POX and CAT (Wang et al. 2001, Samson et al. 2005). Therefore, it is evident from this study that POX levels are elevated to scavenge H_2O_2 and convert it into water and molecular oxygen after ultrasound stress. In addition, POX may have been the main antioxidant enzyme metabolizing H_2O_2 in CAT and POX in this study. This result supports the reports that POX activity is more important than CAT for destruction of H_2O_2 in rat brains (Manikandan & Devi 2005).

In summary, the present study contributes to our understanding of oxidative stress on adult, pupae, and larvae of *H. armigera* following ultrasound exposed. Our experimental data show that ultrasound-stress effects on the antioxidant enzymes system in different developmental stages of *H. armigera* differ significantly depending on the duration of exposure. It provided evidence that oxidative damage occurs in *H. armigera* after ultrasound-stress.

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