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Expression of the Hsp40, Hsp70 and Hsp90 proteins in Colorado Potato Beetle (*Leptinotarsa decemlineata* Say) After the Dimethoate Treatment

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ABSTRACT

Hsp40, Hsp70 and Hsp90 proteins exhibit increased level of expression in animals exposed to negative environmental conditions, including pesticides, but the measured effect depends on the class which particular Hsp is belonging to. In this study we examined Hsp level in the Colorado potato beetle exposed to pesticide dimethoate by using Western blotting and ELISA method. The highest level of expression was exhibited by Hsp90, while Hsp70 showed the lowest level of expression. There were no statistically significant differences in the expressions of Hsp40 and Hsp70 after the pesticide treatment. The highest concentration of Hsp90 was found in beetles 24 hours after the exposition to dimethoate, while the lowest was observed in insects 1 hour after the treatment. Hsps play a role in response to organophosphorous pesticide in *Leptinotarsa decemlineata* (Say, 1824), but there is not any simple correlation between Hsp levels and time of exposition. Hsp90 seems to be the most suitable protein to examine pesticide-provoked stress.

Key words: Colorado potato beetle, heat shock proteins, dimethoate, Hsp40, Hsp70, Hsp90

INTRODUCTION

Colorado potato beetle CPB - *Leptinotarsa decemlineata* (Say, 1824) is a Coleoptera species belonging to the leaf beetle family Chrysomelidae. CPB preyed on several species belonging to the family Solanaceae for many years with no economic significance, for example on *Solanum rostratum*. However, that situation changed after the beginning of potato (*Solanum tuberosum*) cultivation in America by European settlers and farmers. Newly arrived plant proved to be much more abundant in nutrients and more available, therefore Colorado beetle has changed its eating habits and has been seen for the first time on potato crops in 1859. Since that time, beetle began to spread rapidly across the American continent and later

across Europe. Currently, CBP is observed on the area of about 16 million km^2 on two continents (North America and Eurasia) and continues spreading. In Poland beetle appeared probably in 1944, entered from Germany with host plant and since then, it has become the most serious pest of potato crops (Węgorek, 2003, 2007; Alyokhin *et al.*, 2008; Kałmuk and Pawłowski, 2008).

CPB exhibits all of known resistance mechanisms against pesticides that occur in insects. They are mainly related with physiology and/or behavior modifications, including enzymatic detoxification mechanisms, involving oxidoreductases (mainly monooxygenases or cytochrome P450 isoforms), hydrolases (such as carboxylesterase and phosphotriesterases) or transferases (mainly glutathione S-transferases and glucosyltransferases) and reduction of nerve tissue sensitivity in pesticide target places, for example by mutations in the genes encoding acetylcholinesterase enzymes (AChE) or sodium channels, as well as the presence of an additional lipoprotein layer to protect nerve cells (Węgorek, 2007; Alyokhin *et al.*, 2008).

Dimethoate (O,O-dimethyl S-(2-(methylamino)-2-oxoethyl) dithiophosphate) is an insecticide belonging to the group of the organophosphorus compounds. Like other organophosphorus insecticides, it is a strong neurotoxin, which acts by inhibiting acetylcholinesterase enzyme in nervous system (Walker *et al.*, 2001). Dimethoate was introduced in 1956 and it has been used in developed countries on a large scale, mainly in orchards, against crop pests and also against house fly (*Musca domestica*). Dimethoate enters to the insect body with food, by inhalation or by cuticle. It is a toxic compound for many invertebrates, for example to honeybees (*Apis mellifera* L.) (Fischer *et al.*, 1997; Augustyniak *et al.*, 2007). In Poland dimethoate is recommended insecticide for the control of Colorado potato beetle populations (Węgorek, 2007).

Heat shock proteins (Hsps) constitute 5-10% of all proteins present in the cell. Their location depends mainly on their affinity to cell organelles or substrates. Under normal conditions, they accumulate mostly in the endoplasmic reticulum, but also in mitochondria and ribosomes. They are also associated with the cell cytoskeleton, such as microtubules and microfilaments or centrosomes during cell multiplication. Under stress conditions increasing level of Hsps in cytoplasm and their transport to the nucleus is also observed, where they protect DNA, pre-mRNA and nuclear proteins from degradation and damages, furthermore they are also involved in the activation of specific genes (Kaźmierczuk and Kiliańska, 2009). Studies showed that the Hsps expression is induced by many factors such as changes in temperature, pH level, osmolarity, radiation and high concentrations of heavy metals, ethanol, antibiotics, fatty acids and reactive oxygen species. The cellular response to stress can also be induced by changes in atmospheric pressure, lack of nutrients and infection by other organisms (Kopeček *et al.*, 2001).

Expression of Hsps after the pesticide treatment depends on several related factors, mainly on: type of pesticide, dose, tissue and type of specific protein (Bagchi *et al.*, 1996; Pyza *et al.*, 1997). Organophosphorus insecticides could, although not necessarily, induce the expression of Hsp proteins (Bagchi *et al.*, 1996). Experiments also show that even if the stress protein is expressed, the amount thereof is not

fully correlated with the toxic dose of used compound (Pyza *et al.*, 1997). However, there are also studies showing that there is a close correlation between the level of expression of stress proteins and the amount of used pesticide (Bagchi *et al.*, 1996).

Regarding the above mentioned role of Hsp in organism response to pesticide intoxication as well as the lack of information concerning Colorado potato beetle, the aim of this study was to examine Hsp level in *L. decemlineata* subjected to dimethoate in the laboratory conditions.

Our research hypothesis presupposes that biosynthesis of stress proteins: Hsp40, Hsp70 and Hsp90 will increase as a result of the participation of these proteins in the cellular response to stress induced by dimethoate in laboratory conditions in *L. decemlineata*.

MATERIALS AND METHODS

Beetles and laboratory treatment

Adult individuals of the Colorado potato beetle CPB (*Leptinotarsa decemlineata* Say) were hand-collected through direct searching on the plants in August 2011 in Myszków (Silesian Upland, Poland) from cultivated potato fields. Insects were weighted and divided into the control and pesticide groups in the laboratory (Table 1). Beetles from pesticide groups were exposed to dimethoate (BI 58 NOWY 400 EC, BASF SE, Germany). In the case of the group exposed to the pesticide, five insects were slightly anaesthetized on ice and treated with 0.65 µl of insecticide solution (topical application 0.15 µg a.i. per 100 mg of fresh weight) for 1 hour (P1 group) and 24 hours (group P24) at room temperature on a Petri dish without food. To each pesticide group control individuals were treated at the same time an identical volume of distilled water. After the experiment all treated beetles which survived were placed in Eppendorf tubes and frozen at -70 °C. The pilot experiment showed no significant statistical differences between groups: C, C1 and C24, which is why it was decided to unite these groups into one larger control group C.

Sample preparation and measurements

Before analyses of Hsps content, the beetles were homogenized in an ice-cold 0.1 M PBS (7.4 pH) with additives (mercaptoethanol, preoteases inhibitor PMSF - phenylmethylsulfonyl fluoride and NaN_3). To facilitate the process of homogenization the heads, legs, wings and wing covers of each insect were manually removed. The homogenates were centrifuged for 10 minutes at 4°C, 15000 g. The supernatants were collected and frozen at -20°C for further assays. Determination of the amount of protein was performed by the Bradford method (Bradford, 1976) using BSA (Fluka) as a standard.

ELISA test

Hsp content was determined by indirect ELISA protocol (Crowther, 2009) optimized according to Pyza *et al.* (1997) with modifications of Chavez-Crooker *et al.* (2003).

Micro titer plates (96 Well Flat Bottom, Costar 3370) were coated with 100 µl of homogenates with known and identical protein concentration overnight at 4°C. Then, homogenates were removed from the wells and washed three times in TBST (0.1M TBS buffer with 0.05M Tween® 20, Calbiochem). Then active sites remaining on the plates were blocked with 1% BSA to eliminate nonspecific binding of antibodies. The incubation lasted one hour and was carried at 37°C. After the series of triple washing (in TBST), 100 µl solution of the primary antibody anti-Hsp70/Hsc70 mouse pAb (Calbiochem), diluted thousand times in homogenization buffer, was added. Incubation with the primary antibody lasted 2.5 hours at 37°C. After this time residues of the antibody solution were removed by washing three times with TBST, 10 minutes each. Then, secondary antibody (Rabbit Anti-Mouse IgG H&L Chain Specific Alkaline Phosphatase Conjugate) was added, diluted x1000. Incubation took place at 37°C and lasted two hours. After that time, the wells were washed three times with 100 µl of TBST, 10 minutes each. After completing that procedure, solution of p-nitrophenyl phosphate (PNPP, 1mg/ml) in the diethanolamine buffer (pH 9.5) was added. PNPP dye solution induces color reaction in contact with the bounded antibodies. After 30-minute incubation in room temperature, the absorbance level was read in TECAN Infinite M200 Microplate reader (wavelength equal to 405 nm) and expressed as a mean optical density. Several wells with antigen or primary antibody omitted were used to blank the reader.

The same procedure was also performed in the case of ELISA test for Hsp90 and Hsp40 using appropriate antibodies. To detect Hsp90 primary antibody Anti - Hsp90α Rabbit pAb and secondary Goat Anti-Rabbit IgG H & L Chain Specific Alkaline Phosphatase Conjugate Adsorbed were used. For Hsp40 detection primary antibody was Anti-Hsp40 Rabbit pAb and secondary Goat Anti-Rabbit IgG H & L Chain Specific Alkaline Phosphatase Conjugate Adsorbed. For the purposes of calculation and statistics results from all controls (C, C1 and C24) were added into larger group (C).

Western-blot (WB) test

For expression analysis of Hsp40, Hsp70 and Hsp90 proteins Western-blot analyses were also performed. For those tests, volume of the homogenate of each samples had an identical amount of proteins. Samples were supplemented with homogenization buffer to receive identical volume of all. Operations were performed on ice, and after proteins were denatured in water bath for a period of five minutes at 100°C. Homogenate of proteins were loaded and separated by SDS-PAGE (10% polyacrylamide gel containing sodium dodecyl sulfate) for 30 min at 90 V, then 60 min at 120 V, and then transferred to nitrocellulose membrane (HybondTM-C Extra, Amsterdam Biosciences) for 120 min at 150 V (300 mA) with Mini Transfer-Blot (BIO-RAD) apparatus at 4°C. After transferring, the membrane was blocked for 1 h with 3% BSA in Tris-buffered saline (TBS, 150 mM NaCl, 50 mM Tris, pH 7.4), and incubated with primary antibody (Anti-Hsp90α Rabbit pAb; Anti-Hsp70/Hsc70 Mouse pAb; Anti-Hsp40 Rabbit pAb, (Calbiochem) against Hsp90, Hsp70 and Hsp40, respectively. Antibodies were diluted 1:20,000 in 1% BSA in TBS. Incubation was

provided overnight at 4 °C with continuous shaking. Then the membranes were washed three times for 10 min in TBS with Tween-20 (TBST, 150 mM NaCl, 50 mM Tris pH 7.4, 0.1% Tween-20) and incubated with secondary antibody (Goat Anti-Rabbit IgG, H & L Chain Specific Alkaline Phosphatase Conjugate Adsorbed [Calbiochem] for Hsp90, Hsp40 and Rabbit Anti-Mouse IgG, H & L Chain Specific Alkaline Phosphatase Conjugate [Calbiochem] for Hsp70, respectively). Antibodies were diluted 1:3000 in 1% BSA in TBS. Incubation was provided 1 h at room temperature with continuous shaking. After washing (3 x 10 min in TBST), the antibody complex was visualized by colorimetric substrate (BCIP/NBT, 5-bromo-4-chloro-3-indolyphosphate/Nitroblue Tetrazolium), washed again in distilled water and dried. The grey value intensity of the Hsps bands was quantified by densitometric image analysis (Gelscan for Windows version 2.0 software; Kucharczyk, Poland). Relative amounts of Hsps were expressed as mean pixel density per 20 µg protein. As reference for Hsp70 content Hsc70/Hsp73 Protein (bovine, recombinant, Enzo Life Sciences) were used.

Statistics

Obtained results were analyzed using STATISTICA® package (StatSoft, Inc. [2011], STATISTICA, version 10.0. www.statsoft.com). Significant differences in protein expression levels were determined by ANOVA (posthoc RIR Tukey's test). Results with $p < 0.05$ were considered significant.

RESULTS

The mortality rate of Colorado potato beetle was zero in all experimental groups (C, P1 and P24).

ELISA analysis

Exposure CPB to dimethoate caused time-dependent variations in the expression of Hsps, the pattern of the reaction depended mainly on protein family (Table 2, Fig. 1). There were no statistically significant differences in the expression of Hsp40. Using of Hsp70 protein standard allowed the determination of the protein concentration in the tested samples and consequently, percent of total protein in each test group. However, the differences were not significant statistically. The highest concentration of Hsp90 was found in P24 group and the lowest in insects from the group P1 (statistically significant difference). ELISA allows comparing the level of Hsp expression from all studied groups. The highest level of expression exhibited Hsp90, while Hsp70 showed the lowest level of expression.

Western-blot analysis

The amount of proteins contained in a single band was compared with the amount of protein located in the control bands . The molecular weight of the obtained bands and photographic images of nitrocellulose membranes are given below (Figs. 2-4, Tables 3 and 4).

Table 1. Codes of experimental groups and the procedure for their preparation.

Code of experimental group	Number of individuals	Procedure
C	n = 5	frozen
P1	n = 5	cooling on ice (5 minutes); application of pesticide solution; incubation at room temperature (1 hour); cooling on ice (5 minutes); frozen
C1	n = 5	cooling on ice (5 minutes); application of water; incubation at room temperature (1 hour); cooling on ice (5 minutes); frozen
P24	n = 7	cooling on ice (5 minutes); application of pesticide solution; incubation at room temperature (24 hour); cooling on ice (5 minutes); frozen
C24	n = 5	cooling on ice (5 minutes); application of water; incubation at room temperature (24 hour); cooling on ice (5 minutes); frozen

Table 2. The absorbance values reflecting the concentrations of Hsp (mean ± standard deviation) in L. decemlineata subjected to dimethoate and the concentration and percent of total protein of Hsp70 (mean ± standard deviation). The used abbreviations were explained in the Table 1. Superscripts a, b and c describe homogeneity of presented means and when differ between groups there is a statistically significant difference.

Proteins	Experimental group	Mean value of absorbance	SD	Mean value of protein concentration [µg/100µl]	SD	% total protein
Hsp40	C	0,2006 ^{b}	0,0572	-	-	-
	P1	0,2355 ^{bc}	0,0968	-	-	-
	P24	0,2237 ^{b}	0,0525	-	-	-
Hsp70	C	0,1612 ^{b}	0,0121	0,0160	0,0061	0,0535
	P1	0,1683 ^{b}	0,0138	0,0196	0,0069	0,0653
	P24	0,1607 ^{b}	0,0090	0,0158	0,0045	0,0527
Hsp90	C	0,4430 ^{a}	0,1475	-	-	-
	P1	0,3106 ^{c}	0,0786	-	-	-
	P24	0,5016 ^{a}	0,0855	-	-	-

Table 3. The amount of Hsp40 proteins in the bands. Groups description according to Table 1

Amount of material in bands			
Group	C	P1	P24
Band I	100	335,3	431,59
Band II	100	335,3	455,06

Table 4. The amount of Hsp90 proteins in the bands. Groups description according to Table 1.

Amount of material in bands									
	C	C	C	P1	P1	P1	P24	P24	P24
A	0,00	0,00	100,00	94,48	0,00	0,00	132,00	52,08	0,00
B	100,00	221,98	731,12	237,78	419,98	232,59	526,05	631,02	490,7
C	100,00	7,19	140,84	146,17	194,05	500,95	0,00	71,69	2,24
D	100,00	668	246	182,44	81	107	500	543	473

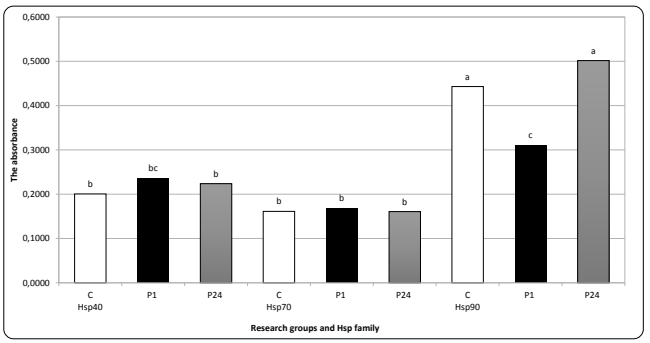

Fig. 1. Absorbance values reflecting the level of expression of Hsp proteins in the L. decemlineata subjected to dimethoate. The used abbreviations were explained in the Table 1. Superscripts a, b and c describe homogeneity of presented means and when differ between groups there is a statistically significant difference.

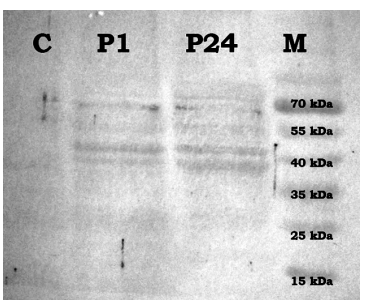

Fig. 2. Nitrocellulose membrane for the Hsp40 protein expression. Groups description according to Table 1. M - molecular weight marker proteins.

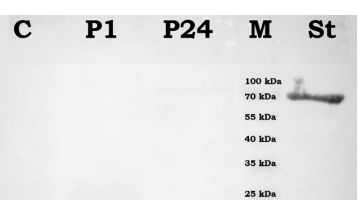

Fig. 3. Nitrocellulose membrane for the Hsp70 protein. Groups description according to Table 1. M - molecular weight marker proteins, St – standard of Hsp70 proteins.

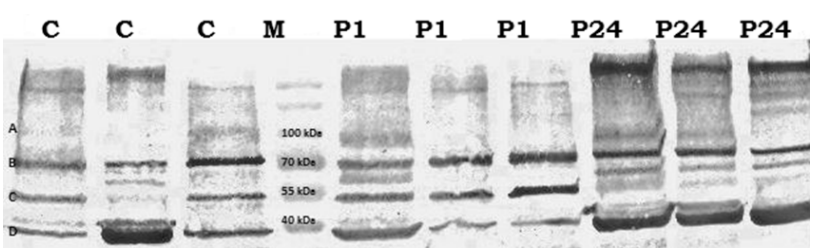

Fig. 4. Nitrocellulose membrane for the Hsp90 protein. Groups description according to Table 1. M - molecular weight marker proteins.

CONCLUSION AND DISCUSSION

As a result of our experiments changes in Hsps levels are probably provoked by indirect dimethoate action, may be via the oxidative stress. There are some studies demonstrating that dimethoate treatment can cause oxidative stress in vertebrates. An example of such experiment is exposure of rainbow trout (*Oncorhynchus mykiss* [Walbaum, 1792]) to 1%, 5% and 10% of the LD_{50} doses . Increase in activity of superoxide dismutase (SOD), glutathione peroxidase (GPx) and catalase (CAT) in the liver and brain tissues was observed. The pesticide also caused lipids peroxidation and DNA damages. Such changes are biomarkers of oxidative stress in cells (Dogan *et al.*, 2011). It is also known that other organophosphorus insecticides such as fenthion or chlorpyrifos, like dimethoate, cause oxidative stress (Bagchi *et al.*, 1996). The stress of this type is responsible for proteins, lipids and DNA damages. Accumulation of oxidative products impairs the functioning of the cells, which can lead to death. Oxidation of -SH groups present in proteins and aromatic amino acid damages by reactive oxygen species (ROS) causing loss of its biological activity (Kulbacka *et al.*, 2009). One of cell defense mechanisms against ROS is reactions involving Hsp proteins (Bagchi *et al.*, 1996; Kulbacka *et al.*, 2009). This may explain the higher level of their biosynthesis, because of their participation in the repair of damaged proteins and preventing their aggregation in non-native form (Kaźmierczuk and Kiliańska, 2009).

ELISA method showed no statistical significant differences in Hsps70 expression in beetles from both pesticide groups compared with the insects from control group. On the basis of these results it can be concluded, that Hsp70 proteins do not take significant part in cell response after dimethoate treatment. Similar results were obtained in experiments performed by Pyza *et al.* (1997). Centipedes (*Lithobius mutabilis* [L. Koch, 1862]) were kept for 28 days in soil contaminated by dimethoate, respectively at a dose of 0, 3.1 and 12.5 mg per kg of soil. Next, ELISA and Western-blot were performed to examine level of Hsp70 protein expression, but there was not any statistically significant difference. Similar results have been shown in pupae housefly (*Musca domestica* L., 1758) by using the aforementioned methods in the same experiment (Pyza *et al.*, 1997).

There are studies indicating that Hsp70 proteins are involved in potato beetles diapause. The increase of LdHsp70A protein expression has been shown during diapause period, whereas no increase of LdHsp70B expression has been showed. However, both exhibit high expression level by using a cold shock. Hsp70 proteins may have a significant role in the survival of the beetle during winter period (Yocum, 2001). The level of Hsp70 protein is important also as a factor limiting CPB expansion in the cold areas. Production of Hsp70 is because temperature shock is less intense in CPB which are more frequently exposed to unfavorable temperatures in their habitat than those occupying benign conditions (Lyytinen *et al.*, 2012).

ELISA method detected increase of the Hsp90 proteins expression (about 1.6 times). Also immunoblotting analysis gave similar results. The amount of material in proteins bands in beetles from the group P24 was about 1.4 times higher comparing

with P1 insects, and exhibit molecular weight equal to about 90 kDa,. However, it should be noted that not every path appeared 90 kDa proteins, so it can be assumed that Hsp90 protein biosynthesis is determined by individual factors. However, in each insect there have been many colored bands on the nitrocellulose membrane. There are mainly three additional strong bands, occurring in almost every track, with average molecular weights: 73.35 kDa, 56.65 kDa and 42.16 kDa. This phenomenon is connected with polyclonality of the used primary antibody.

Both (ELISA and WB) methods showed that Hsp90 are the most abundant proteins of Hsps occurring in CPB cells, these proteins are also most abundant occurring stress proteins in eukaryotic cells (Kopeček *et al.*, 2001).

In our work the increase in biosynthesis of Hsp90 proteins was probably caused by an appearance of oxidative stress in the cells, as it was described above. One of the defense mechanisms against oxidative stress involves reactions with heat shock proteins (Kulbacka *et al.*, 2009). This may explain the paired increase of Hsp90 and Hsp40 biosynthesis, as both are involved in the repair of damaged proteins and removal of denatured proteins (Kaźmierczuk and Kiliańska, 2009). In our work we observed also time dependent reaction the level of Hsp90 in beetles exposed to dimethoate for 24 hours was higher than in CPBs exposed for one hour only.

There are studies showing that there is a close correlation between the level of expression of stress proteins from Hsp90 group and the amount of used pesticide. An example of that experiment was exposure of Sprague Dawley laboratory rats (*Rattus norvegicus* [Berkenhout, 1769]) to various types of pesticides, including fenthion and chlorpyrifos. Hsp90 protein expression was investigated by Western Blot method and the expression of Hsp89β and Hsp89α genes by Nothern Blot method. A correlation between the dose of pesticides and Hsp90 expression in the liver, brain and lung of animals was showed. The level of this biosynthesis differed depending on the type of insecticide used and the tissue. This study also determined the level of expression of Hsp90 proteins in PC12 cell culture, wherein the increased level of Hsp89β and Hsp89α gene expression was showed. It can be concluded that expression of Hsp90 depends on the type of toxin and the type of tissue. Expression of these proteins is to protect cells from potential damage arising as a result of the treatment of xenobiotic and oxidative stress (Bagchi *et al.*, 1996).

The decrease of Hsp90 proteins amount from P1 group compared to the control group can be explained by the fact that the Hsp proteins may be directly damaged by pesticide or its derivatives and the cells do not have time for biosynthesis of new, to replace these denatured. Western blot analysis in this case also showed a major drawback of ELISA analysis. Application of molecular proteins marker allows determining the molecular weights of proteins contained in the bands and usage of the standard allows answering the question whether the experience has been done properly and whether the applied antibodies actually connect to the interesting protein (positive control). The analysis of bands also allows determining whether this antibody is specific or not. ELISA result shows only amount of proteins bounded with antibodies

and possibly its concentration, by using the appropriate standard. It is impossible to determine whether the antibodies bound specifically or not.

Cellular response which involves heat stress proteins depends on various factors and results depending on used method. For these reasons, stress protein Hsp can be not regarded as a universal biomarker in ecotoxicological studies and the results of the particular experiments should not be directly transferred onto other organisms. In our paper we demonstrated that Hsps play a role in response to organophosphorous pesticide in *Leptinotarsa decemlineata*, but there is not any simple correlation between Hsps level and time of exposition. Hsp90 seems to be the most suitable protein to examine pesticide-provoked stress.

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