Comparative Biochemistry and Physiology Part C 132 (2002) 105–112

www.elsevier.com/locate/cbpc

Trends in detoxification enzymes and heavy metal accumulation in ground beetles (Coleoptera: Carabidae) inhabiting a gradient of pollution

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Received 26 June 2001; received in revised form 12 November 2001; accepted 11 March 2002

Abstract

Non-specfic carboxylesterase and glutathione *S*-transferase activity was measured in the ground beetle, *Pterosthicus oblongopunctatus* (Coleoptera: Carabidae), from five sites along a gradient of heavy metal pollution. A previous study determined that beetles from the two most polluted sites (site codes OLK2 and OLK3) were more susceptible to additional stressors compared with beetles from the reference site (Stone et al., Environ. Pollut. 113, 239–244 2001), suggesting the possibility of physiological impairment. Metal body burdens in ground beetles from five sites along the gradient ranged from 79 to 201 μg/g Zn, 0.174 to 8.66 μg/g Pb and 1.14 to 10.8 μg/g Cd, whereas Cu seemed to be efficiently regulated regardless of metal levels in the soil. Beetle mid- and hindguts were homogenized and the soluble fraction containing glutathione *S*-transferase (GST) and carboxylesterase (CaE) was assayed using kinetic analyses. Significantly higher levels of GST were found only in female beetles from the most polluted sites (OLK2 and OLK3; $P=0.049$, $P<0.001$, respectively) compared with the reference site (OLK7). In addition, OLK3 females had significantly higher levels of CaE compared with the reference beetles ($P=0.01$). Male beetles did not differ in enzyme activity along the metal gradient. Overall, obvious trends in detoxification enzymes were not detected in ground beetles in association with metal body burdens.

Keywords: Glutathione *S*-transferase; Carboxylesterase; Heavy metals; Coleoptera; Carabidae; Biomarker; Stress; Ecotoxicology

1. Introduction

The central question addressed in this study is whether trends in detoxification enzyme activity can be detected in the ground beetle, *Pterostichus oblongopunctatus,* sampled along a heavy metal gradient in Southern Poland. We have previously demonstrated that beetles from the most polluted sites show an accelerated time to death, when exposed to additional stressors (Stone et al., 2001). Since a major focus of ecotoxicology is to assess stressors acting at sub-lethal levels, there has been recent emphasis on developing and validating sensitive biomarkers that do not require assays with large numbers of animals. A promising approach is to measure the kinetic activity of selected enzymes in target organisms (Mitton, 1997). Although the majority of previous studies that reported enzyme activity for terrestrial invertebrates have been carried out to detect insecticide resistance that results from elevated levels of detoxification enzymes (Kirby et al., 1994; Karoly et al., 1996), this study examined enzyme activity

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PII: S1532-0456(02)00052-2

as a biomarker of physiological stress in a chronically polluted environment.

When xenobiotics cause physiological responses, such as enzyme activity, to deviate beyond typical ranges, then individual fitness may be impaired (Calow, 1991; Calow and Forbes, 1998). If exposure to stress decreases enzymatic activity below normal limits, then inhibition has occurred, and assays that determine the level of enzyme inhibition may effectively identify populations that are experiencing stressful conditions.

Enzyme induction above normal ranges may also result in reduced fitness because of the energetic demands imposed (Sibly and Calow, 1989). Organisms are constrained within limited energy budgets and induction of high enzyme levels may decrease fitness by diverting energetic resources from maintenance and reproduction (Berenbaum and Zangerl, 1994). Additional impacts of induction may include greater susceptibility to subsequent stressors and reduced performance in the absence of the selection pressure.

To determine if trends in enzyme expression could be observed, the ground beetle, *Pterostichus oblongopunctatus*, (Coleoptera: Carabidae), was collected along a gradient polluted with cadmium, copper, lead and zinc. *P. oblongopunctatus* were analyzed for body burdens of these heavy metals to quantify accumulation levels. In addition, the enzyme activity of non-specific carboxylesterases (CaE) and glutathione *S*-transferases (GST) were measured in the soluble fraction of gut homogenates. CaEs are Phase I enzymes that react with non-polar compounds through hydrolysis. The resulting metabolites are then further metabolized by Phase II enzymes or excreted. GSTs comprise a family of Phase II isozymes that conjugate electrophilic compounds with reduced glutathione. GSTs play central roles in the detoxification of many xenobiotics (Eaton and Bammler, 1999; Scharf et al., 1999). Furthermore, the level of isozyme expression for both CaE and GST may be modified by exposure to various xenobiotics (Iio et al., 1993).

In a previous study, reduced survivorship was determined for *P. oblongopunctatus* at the two most polluted sites along the gradient of heavy metal pollution compared with the reference site (Stone et al., 2001). The beetles from the two most polluted sites (OLK2 and OLK3) had a significantly lower time to death (TTD) compared with beetles collected from the reference site (OLK7). During this study, beetles were sampled for enzyme analysis at the same sites to determine if a physiological link could be established between metal exposure and increased susceptibility to multiple stressors. The influence of gender on enzyme expression was also analyzed to determine the suitability of GST and CaE as biomarkers of ecotoxicological stress in Carabidae.

2. Materials and methods

2.1. Study sites

The sample sites were located along a gradient of heavy metal pollution in the vicinity of Olkusz, Poland (approximately 50 17′N/19 31′E to 50 32′N/19 39′E). Two zinc ore smelters and several mines are located approximately 4 km apart (consuming approx. 3 million tons of zinc-lead ore annually). Currently, dust emission from these smelters reaches approximately 45 tons per year. In the past, the annual dust precipitation in the region was approximately 118 tons/km^2 resulting in intense soil pollution extending over thirty square kilometers. Five sites (OLK2, OLK3, OLK4, OLK6 and OLK7) were used for research, based upon concentrations of metals in the humus layer. OLK7 is the reference site, which has metals found at background levels. These sites represent a broad range of pollution, ranging from 150 to 10 500 mg kg^{-1} Zn in dry humus, 140–2600 mg kg^{-1} Pb, 0.8–100 mg kg^{-1} Cd and 11–74 mg kg^{-1} Cu (Table 1). The soils throughout the gradient are characterized by acidic, podsolized, sandy types with a well-developed mor humus layer.

Beetles in the sampling sites were collected with pitfall traps (approx. 200 ml capacity). Fifty traps were distributed along two transects at each site. Transects and traps were set at 3 m intervals and emptied every third or fourth day. The beetles were transported to the laboratory in plastic boxes containing soil from collection sites. Within 48 h of collection, the beetles were separated by gender and sample location and frozen at −27 °C.

2.2. Metal analysis

In addition to the beetles collected for enzyme analysis, beetles were sampled for body burdens of copper, zinc, lead and cadmium. These beetles were separated into site and gender and kept in

Table 1
Mean concentrations of four heavy metals ($\pm$S.D.) detected in the humus layer along the heavy metal gradient

Site	Zn (mg kg^{-1})	Cd (mg kg^{-1})	Cu (mg kg^{-1})	Pb (mg kg^{-1})
OLK2	10454	81.9	46.9	2635
	(2618)	(17.2)	(4.6)	(120.4)
OLK3	5104	51.1	37.6	1832
	(729)	(19.3)	(3.7)	(215)
OLK4	1522	18.1	25.6	870
	(135)	(2.6)	(2.16)	(36.3)
OLK6	244	3.3	15.4	355
	(78)	(1)	(2.7)	(30.9)
OLK7	151	0.84	10.7	136
	(35)	(0.4)	(1)	(8.8)

containers for 24 h to void gut contents. Following this period, the organisms for metal analysis were stored at −70 °C until analysis for metal content. Whole beetles were dried at 105 °C, weighed and digested in 1 ml of nitric acid. Concentrations of zinc, copper and lead were analyzed with flame atomic absorption (AAS) and cadmium was analyzed with graphite AAS (Perkin–Elmer, AAanalyst 800). Five males and five females were analyzed for all four metals from each site along the sampling gradient.

2.3. Enzyme analysis

Individual beetles had their entire guts removed and were homogenized in one ml of 1.15% KCl buffer with a few crystals of phenylthiourea. Homogenization was conducted on ice, using a motor-driven pestle. Immediately after homogenization, beetle guts were centrifuged at 10 000 rpm for 10 min. The supernatant was collected (soluble fraction) and stored at −27 °C for less than two weeks. Twelve male and twelve female replicates were homogenized from each site for GST and CaE activity.

GST was analyzed using the method of Habig et al. (1974), Grant et al. (1989) with modifications. The substrate, 1-chloro-2,4-dinitrobenzene (CDNB), was dissolved in DMSO to give a 75-mM concentration of substrate (50 mM final concentration). 305 μl of this solution was added to 20 ml of KPO_4 buffer (100 mM, pH 8.0, containing 15% glycerol). 200 μl of the buffer/substrate solution was pipetted into an individual microtiter plate well, followed by sample homogenate equivalent to 0.07 mg protein. Finally, 30 μl of 8 mM glutathione was added to initiate the reaction (total volume in each well was 300 μl). The change in optical density was measured over the initial 10 min of the reaction at 340 nm and 30 °C. GST was corrected for non-enzymatic activity by subtracting blanks (buffer and GSH only) and the results were converted to specific activity in units of nmoles min^{-1} mg $protein^{-1}$ using an extinction coefficient of 10.9 mM^{-1} 300 μl^{-1} (Grant et al., 1989). Two to three replicates were run for each sample.

CaE was measured using the substrate α-naphthyl acetate (α-NA) as outlined by Gomori (1953) and modified by Grant et al. (1989). Substrate solution was prepared by dissolving 18 mg of Fast Blue B salt in phosphate buffer (100 mM, pH 7.0). To this solution 600 μl of 0.113 M α-NA dissolved in 50% acetone was added. 240 μl of substrate solution was pipetted into microtiter wells after filtration (Whatman No. 3). 10 μl of homogenate was added for a total volume of 250 μl per well. The change in optical density was monitored for the initial 10 min at a wavelength of 450 nm and 30 °C. Results were corrected for non-enzymatic activity by subtracting blanks (buffer) and converted to units of nmoles min^{-1} mg $protein^{-1}$ using an extinction coefficient of 9.25 mM^{-1} 250 μl^{-1} (Grant et al., 1989). Two to three replicates were run for each sample.

The amount of protein in the samples was estimated by the method of Bradford (1976), using bovine serum albumin (fraction V) as the standard.

2.4. Statistical analyses

The data were examined for differences among locations, between genders and for the interaction between gender and site, taking into account any differences in fresh body weight. The data were tested for homogeneity of variance using Levene's

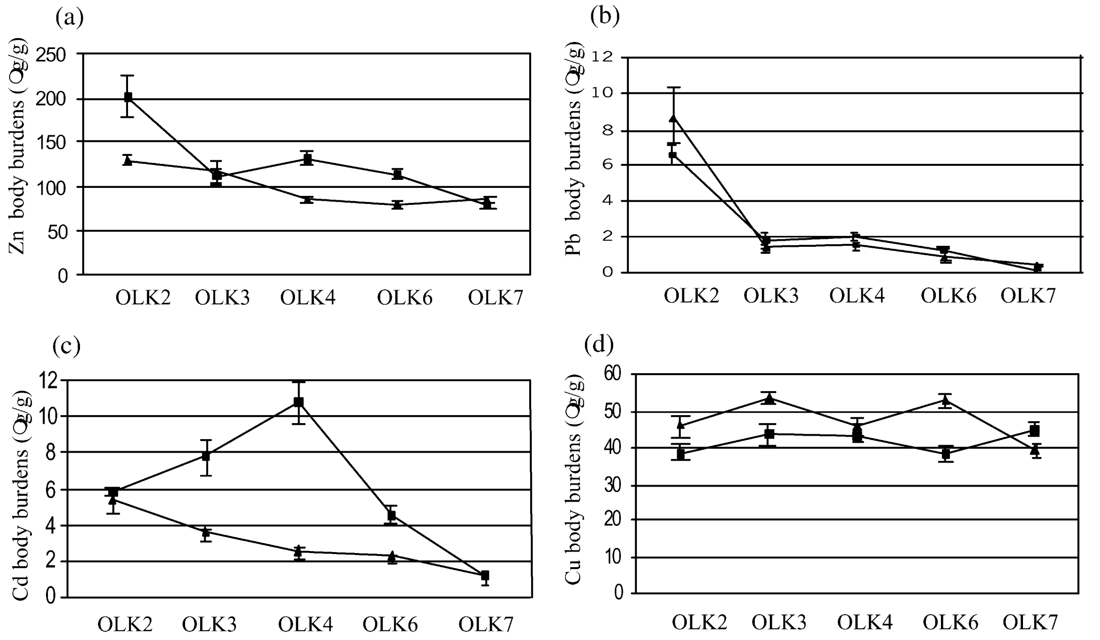

Fig. 1. Concentrations of (a) zinc, (b) lead, (c) cadmium and (d) copper in bodies of *P. oblongopunctatus* along a heavy metal gradient (squares = females, diamonds = males). Error bars represent standard error.

Test of Equality of Error Variances. A general linear model (GLM) was conducted to determine if significant heterogeneity in enzyme expression existed with respect to site and gender. If significant heterogeneity ($P \leq 0.05$) was detected, Tukey's multiple comparison test was used for pair-wise comparisons. Statistical analysis was conducted using SPSS version 8.0.

3. Results

The average concentrations of zinc ± S.E. were 201 (±11.8), 112 (±23.1), 131 (±13.9), 114 (±10.6) and 79 (±7.6) in female carabids and 130 (±12), 118 (±14), 86 (±5.2), 81 (±2.3) and 86 (±11.2) in male carabids collected from OLK2, OLK3, OLK4, OLK6 and OLK7, respectively (Fig. 1a). Average lead concentrations were 6.5 (±0.9), 1.8 (±0.6), 1.9 (±0.18), 1.3 (±0.36) and 0.17 (±0.1) in female carabids and 8.7 (±3), 1.4 (±0.14), 1.5 (±0.18), 0.8 (±0.17) and 0.36 (±0.1) in male carabids collected from OLK2, OLK3, OLK4, OLK6 and OLK7, respectively (Fig. 1b). Average cadmium concentrations were 5.8 (±0.4), 3.8 (±1.9), 10.8 (±2.3), 4.6 (±0.7) and 1.3 (±0.3) mg/kg DW in female carabids and 5.4 (±0.4), 3.6 (±0.6), 2.6 (±0.6), 2.3 (±0.4) and 1.2 (±0.3) mg/kg DW in male carabids collected from OLK2, OLK3, OLK4, OLK6 and OLK7, respectively (Fig. 1c). Average copper concentrations were 38.3 (±3.5), 43.6 (±5.6), 43.2 (±10.8), 38.1 (±4.9) and 44.6 (±3.9) mg/kg DW in female carabids and 46.4 (±5.6), 53.6 (±3), 45.8 (±4.4), 52.9 (±4.2) and 39.3 (±4) mg/kg DW in male carabids, collected from OLK2, OLK3, OLK4, OLK6 and OLK7, respectively and (Fig. 1d).

For glutathione *S*-transferase, the average nmoles of product formed min^{-1} mg protein^{-1} ranged from 150 nmoles in the reference site to 200.6 nmoles in OLK3. Levene's Test demonstrated that variance in enzyme activity did not differ significantly between the sample sites in either males ($P = 0.568$), females ($P = 0.542$), or both genders in combination ($P = 0.721$). Significant differences among enzyme activity was detected between sites ($P < 0.001$), genders ($P < 0.001$) and for the interaction between gender and site ($P = 0.017$), requiring the genders to be tested separately for differences in GST activity.

No significant differences between sites in GST activity among males collected along the sample gradient ($P = 0.641$, Fig. 2). GST activity in females differed significantly between sample sites ($P < 0.001$), ranging from 118.4 nmoles in the

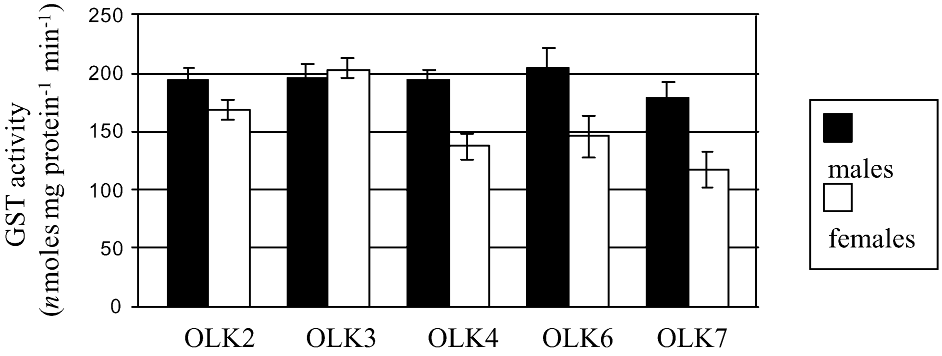

Fig. 2. GST activity in *P. oblongopunctatus* males and females collected from sites along the heavy metal gradient. Error bars represent standard error.

reference site (OLK7) to 204.2 nmoles product formed min^{-1} mg protein^{-1} in OLK3. Tukey's multiple comparison test detected significant differences between OLK2 and the reference site ($P=0.049$) and OLK3 and the reference site ($P<0.001$, Fig. 2). The trend for females was for the enzyme activity to be higher in the more polluted sites.

For non-specific carboxylesterases, the average nmoles of product formed min^{-1} mg protein^{-1} ranged from 71.3 nmoles in the reference site to 107.5 nmoles product formed min^{-1} mg protein^{-1} in OLK3. Levene's Test demonstrated that the variance in enzyme activity was not significantly different along the sample gradient in males ($P=0.253$), females ($P=0.05$) or for both genders in combination, ($P=0.105$). Enzyme activity differed significantly between sites ($P<0.001$) and that the interaction between gender and site was also significant ($P<0.001$). The genders were therefore, analyzed separately for CaE activity.

No significant difference in CaE activity among males between sites ($P=0.091$, Fig. 3), although the difference approached the significance level chosen for this study ($P=0.05$). CaE activity in females however, differed significantly between sites ($P<0.001$). Females ranged in CaE activity from 62.7 nmoles in OLK4 to 103.6 nmoles product formed min^{-1} mg protein^{-1} in OLK3. Tukey's multiple comparison test detected significantly higher expression between OLK3 and the references site ($P=0.01$) and between OLK3 and OLK4 ($P=0.003$, Fig. 3).

No significant differences in weight were detected among males or among females between sample sites along the gradient (one way ANOVA, $P=0.237$). Average weights of female beetles (±SD)

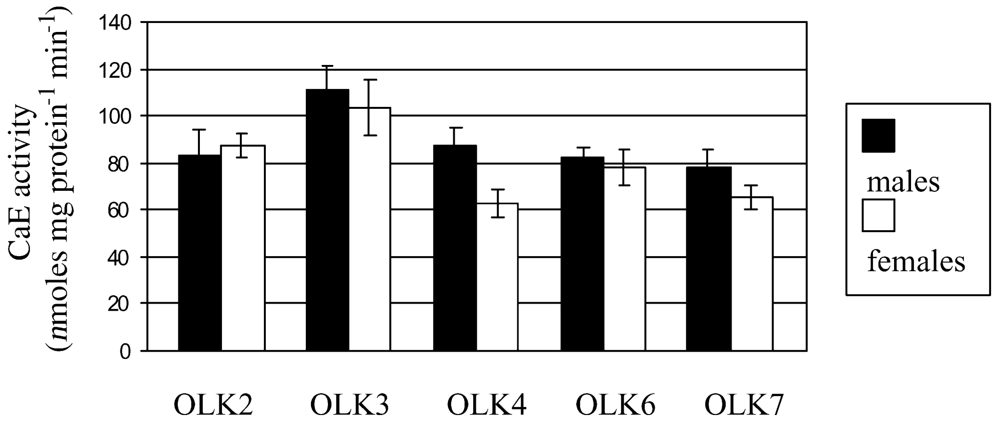

Fig. 3. CaE activity in *P. oblongopunctatus* males and females collected from sites along the heavy metal gradient. Error bars represent standard error.

from each sample site were 58.6 (4.5) mg at OLK2, 59.9 (5.1) mg at OLK3, 60.3 (5) mg at OLK4, 56.3 (8.1) mg at OLK6, and 55.2 mg (6) at OLK7. The average weight of male beetles (±SD) was 51.9 (4.5) mg at OLK2, 44 (5.7) mg at OLK3, 53.9 (4.6) mg at OLK4, 46.3 (8.2) mg at OLK6, and 50.7 (2.8) mg at OLK. However, significant differences in weight were detected between males and females (two sample *t*-test, $P=0.005$), further emphasizing the need to segregate gender in this study.

4. Discussion

Theoretically, glutathione *S*-transferase (GST) and carboxylesterase (CaE) are ideal biomarkers of ecotoxicological stress. They are ubiquitously distributed in the biota, rapidly and reproducibly measured, inducible, and play active roles in the detoxification of endogenous and exogenous compounds. Not surprisingly therefore, GST and CaE have been investigated as biomarkers in terrestrial invertebrates collected from chronically polluted environments. Wilczek et al. (1997) found that spiders collected from metallurgic dumps in the Czech Republic had higher levels of GST activity compared with spiders from reference sites. CaE activity, however, was generally lower in coal-polluted sites compared with the control site. Both enzymes demonstrated time activity differences measured over two years. In another study, wolf spiders had elevated levels of CaE and GST from polluted regions of Southern Poland (Wilczek and Migula, 1996). In contrast, no effect on GST level was observed in the bloodworm, *Eisenia fetida,* after accumulation of lead, zinc and cadmium (Grelle and Descamps, 1998). These conflicting results prompted us to analyze the utility of detoxification enzymes as indicators of physiological stress in beetles exposed to chronic pollution.

The present study observed that female beetles from OLK3 had significantly higher expression of GST and CaE compared with females from the reference site (OLK7), and females from OLK2 had a significantly higher level of GST activity compared with OLK7. In contrast, no significant trends in activity were detected in males for either CaE or GST across the sample gradient. Surprisingly, the most elevated levels of enzyme activity were found in male and female carabids collected at OLK3. While highly polluted, the soil concentrations of Zn, Cd, Pb and Cu are not as high as OLK2. This observation alone would cast skepticism on the utility of detoxification enzymes as biomarkers of sub-lethal metal exposure and seems to suggest an unrelated or confounding factor responsible for the elevated CaE and GST activities found at OLK3. However, Stefanowicz (2001) measured a considerably lower pH at OLK3 compared with OLK2 (5.6 vs. 6.7). This resulted in a significantly higher concentration of water soluble Zn at OLK3 (15.9 mg/kg) compared with OLK2 (8.4 mg/kg). Therefore, even though the total Zn concentrations are nearly twice as high as OLK2, the water-soluble fraction is higher at OLK3 and may impose greater physiological effects on the beetles.

In general, males had higher levels of both enzymes per gram body weight, compared with females and males did not significantly fluctuate in enzyme activity between sites. Perhaps detoxification enzymes are more inducible in female carabids exposed to physiological stress, while males have higher constituent levels. Alternatively, females may be more susceptible to fluctuations in enzyme levels as a result of reproductive physiology, lipid deposition, diet or other processes operating independently from responses to metal exposure. These possibilities warrant further scrutiny if detoxification enzymes can be developed as reliable biomarkers of sub-lethal stress.

Differences in GST and CaE activity among genders have been reported for other invertebrates (Almar et al., 1987). In addition, age specific (Kedziorski et al., 1996; Kostaropoulos et al., 1996) and tissue distribution differences (Konno and Shishido, 1992) of detoxification enzymes have been observed in terrestrial insects. Chrascina et al. (1996) found a decrease in gut levels of GST in the caterpillar, *Smerinthus ocellatus,* while levels were slightly induced in fat bodies after exposure to cadmium in the diet. The physiological differences in absorption, distribution, metabolism and excretion, as well as the differing life history attributes among taxa are not well understood and may impair extrapolations from individual studies to more general patterns.

Although carabids are characterized as poor accumulators of heavy metals (Kramarz, 1999; Heikins et al., 2001), distinct trends were found in some of the metals analyzed. Zinc levels accumulated noticeably at the most polluted site (OLK2) compared with other sites along the gradient, with the highest levels found in female carabids (Fig.

1a). Lead was found in the highest levels at OLK2, but did not accumulate appreciably in relation to concentrations found in the environment (Fig. 1b). The lack of lead accumulation in beetles (<10 ppm at OLK2) in relation to the concentrations found in the environment (>2500 ppm) has been previously documented (Bengtsson and Rundgren, 1984; Beyer et al., 1985). Cadmium displayed the greatest differences between genders. Male carabids showed an expected pattern of accumulation along the pollution gradient. Females, however, accumulated the most cadmium at OLK4, followed by OLK3 and OLK2 (Fig. 1c). Females may have been feeding on different prey items across the sample gradient. For instance, the females at OLK4 may have consumed prey that readily bioaccumulated metals easily. In these circumstances, high accumulators may have been absent from the most polluted sites because their uptake becomes detrimental, and these species would no longer be consumed. Lindqvist and Block (1998) observed that Cd and Zn accumulation rates tended to be linked in female beetles and not males, adding further complexity to multiple pollutant studies. Copper levels fluctuated slightly among the sampling sites with no apparent trends (Fig. 1d). This may indicate that copper is efficiently regulated at the more polluted sites or that uptake is reduced.

In this investigation, the enzyme activity that was most promising for its potential use as a biomarker was the elevated expression of GST noted in females from the two most contaminated sites compared with the reference site. CaE expression was less reliable as a biomarker, as evident in elevated activity for OLK3 females but not OLK2 females compared with the reference site. Males did not exhibit variation in enzyme activity along the gradient, despite having variable rates of metal body burdens. The challenges involved with interpreting physiological data, such as enzyme activity, with exposure to stress under field conditions are complex. Accordingly, a clear relationship between differential survivorship (Stone et al., 2001), metal accumulation and enzyme activity was not established. However, since physiological fitness is intimately linked with organism and population level scales, these challenges are worth investigating.

Acknowledgments

We are grateful to Mr Maciej Maryanski, MSc, Ms. Maria Niklinska, MSc, Dr Paulina Kramarz and Dr Zbigniew Dabrowski for their invaluable help in the field and laboratory. David Stone was funded by NIEHS Grant #ES07 and visiting Jagiellonian University under the NATO Linkage Grant No. ENVIR.LG 972736.

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