An Abstract of the Dissertation of

David L. Stone for the degree of Doctor of Philosophy in Toxicology on December 19, 2001. Title: Physiological and Organism Level Endpoints in the Beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae), Inhabiting a Gradient of Heavy Metal Pollution.

Redacted for privacy

Abstract approved: _____

Paul Jepson

Pollutant body burdens, ability to tolerate supplementary stressors, and biomarkers of physiological stress were investigated in the ground beetle, *Pterostichus oblongopunctatus* (Coleoptera: Carabidae), inhabiting a gradient of heavy metal contamination in Poland. The central question was to determine if beetles inhabiting polluted habitats incurred costs compared with beetles in less contaminated habitats. Metal body burdens in beetles ranged from 79 to 201 μg/g zinc, 0.174 to 8.66 μg/g lead, and 1.14 to 10.8 μg/g cadmium. Copper was efficiently regulated along the pollution gradient. Beetles from different sites were subjected to supplementary stressors (food deprivation and exposure to the organophosphorous insecticide, dimethoate). Beetles originating from the most contaminated sites (OLK2 and OLK3) were significantly less tolerant of food deprivation (measured as time to death) compared with beetles from the reference site (OLK7). Beetles from OLK2

and OLK3 were significantly more susceptible to dimethoate exposure (median survival times of 12 and 123 hours, respectively) compared with beetles from the reference site (359 hours). There was a negative correlation between chronic pollution burden and ability to survive additional stress. Trends in the enzyme activity of carboxylesterase and glutathione S-transferase (GST) in response to metal exposure were determined for beetles along the gradient. Significantly higher levels of GST were found in female beetles from OLK2 and OLK3 ($p=0.049$ and $p<0.001$, respectively) compared with the reference site. Male beetles did not differ in enzyme activity along the gradient. There was no direct correlation between enzyme activity and exposure to metals. Respiration rate, recorded as CO_2 expiration, was also measured in beetles along the gradient. Beetles collected from OLK2 exhibited significantly lower respiration rates compared with other sites. Changes in respiration rate after challenge with dimethoate were measured to determine physiological responses following exposure to stress. After dosing, respiration rates increased significantly at all sites ($p<0.0001$), suggesting that chronic metal exposure did not impair the ability of beetles to increase respiration rate after dimethoate challenge. While clear costs of metal exposure were found at the organism level, there was no strong correlation that these costs placed beetles at their physiological limits to respond to additional stressors.

Physiological and Organism Level Endpoints in the Beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae), Inhabiting a Gradient of Heavy Metal Pollution

by

David Stone

A DISSERTATION

submitted to

Oregon State University

In partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented December 19, 2001
Commencement June 2002

Doctor of Philosophy dissertation of David L. Stone presented on December 19, 2001

APPROVED:

Redacted for privacy

Major Professor, representing Toxicology

Redacted for privacy

Chair of Department of Environmental and Molecular Toxicology

Redacted for privacy

Dean of Graduate School

I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Redacted for privacy

David L. Stone, Author

Acknowledgements

Several people share credit for this dissertation, ranging from my childhood years to my seemingly endless career as a student. My deepest gratitude is given to my parents, Tamara Durstine and Leonard Stone. Thank you for supporting the paths I've taken in life, providing me with love and encouragement, and guiding me in the right direction. To my siblings, Remy, Bobby and Daniel: you are my greatest inspirations and the best friends a guy could ask for. I'd also like to acknowledge my extended family: Debra Stone, David Durstine, Jennifer Pertle and Darren Spreeuw, and my new family, Ronald, Beverly and Todd Winters. You all have generously accepted my passion for bugs and science, even if you think I'm a little weird.

My academic fortunes have benefited from many contributors inside and outside the laboratory. First, I would like to extend my profound appreciation to my major professor, Paul Jepson, whose enthusiasm for ecotoxicology motivated me to pursue this dissertation and whose guidance kept me on track. Second, Ryszard Laskowski, I am indebted for all you've taught me and the beers we shared in Poland. I also want to thank the Entomology and Ecotoxicology groups of Oregon State University (especially Phil Henneghan), my wise committee members, the National Institute for Environmental Health Sciences for funding me (especially Dr. David William's pivotal role) and the good folks at Jagellionian University in Poland (especially Paulina Kramarz). Also, many great friends helped sustain my sanity along the way, especially Greg Aymond, Mark Russell and James Newell.

Finally, I'd like to acknowledge all the frogs, bugs and other small critters of Pebble Creek, in Dallas, Texas. For years, I collected them, or simply watched them go about the business of life. When the banks of Pebble Creek were developed and the waters began to shine like oil, the diversity of my playground vanished. I began to wonder why they disappeared and without knowing it, launched my path in life.

Contribution of Authors

Dr. Ryszard Laskowski was involved with many aspects of this dissertation. I first collaborated with Dr. Laskowski during our studies on population growth measures in pea aphids. Through our work together, we formed a basis for continued research. I spent two summers at Dr. Laskowski's lab in Krakow, Poland. He assisted in collecting sample organisms, experimental design, data handling and analysis of results. In addition, Dr. Paulina Kramarz assisted in sampling, collection of data and maintenance/culturing of beetles. Both Dr. Laskowski and Dr. Kramarz were essential to my successful endeavors in Poland, both professionally and culturally.

Table of Contents

Page

Table of Contents (Continued)

Page

List of Figures

List of Tables

List of Appendix Figures

List of Appendix Tables

Dedication

I dedicate this dissertation to my wonderful wife and colleague, Kerri Winters Stone.

Thank you for giving your heart so openly and lending your mind so wisely.

Physiological and Organism Level Endpoints in the Beetle *Pterostichus oblongopunctatus* (Coleoptera: Carabidae), Inhabiting a Gradient of Heavy Metal Pollution

Introduction

Ecotoxicology is a science that seeks to understand and predict the effects of chemical stressors on organisms at several levels of biological organization. One of the major assumptions of ecotoxicology and stress ecology is that survival and persistence in chronically polluted environments is costly to the individual (Hoffman and Parsons, 1989; Sibly and Calow, 1989). This dissertation addresses the costs associated with a gradient of heavy metal contamination that is inhabited by the ground beetle, *Pterostichus oblongopunctatus* Fabricius (Coleoptera: Carabidae). The endpoints that were used to measure the potential cost of exposure to these pollutants in *P. oblongopunctatus* include pollutant uptake, differential survival and a suite of physiological biomarkers, including enzyme induction and respiration rate.

P. oblongopunctatus was selected as the test organism for numerous reasons. It was the most abundant predatory invertebrate collected along the metal gradient that also occurred at each sample site. It lacks high dispersal ability relative to the scale of pollution, as a result of underdeveloped wings (Brunsting, 1981). Therefore, this species would be confined to local zones of

contamination over multiple generations. In addition, *P. oblongopunctatus* was easy to maintain in the laboratory, provided ample tissue for physiological assays and was an excellent specimen for topical dosing with insecticide.

The field sites from which *P. oblongopunctatus* were collected are located along a gradient of heavy metal pollution in southern Poland. The pollutants of concern are zinc, cadmium, lead and copper. These metals are persistent, inorganic xenobiotics that can exert negative effects on chronically exposed organisms. The source of pollution is a zinc smelter, with emissions that have caused massive soil pollution over thirty square kilometers (Kapeja et al., 1990). In total, five sites were sampled using pit fall traps. The most polluted sites were designated OLK2 and OLK3. Sites OLK4 and OLK6 had intermediate levels of soil contamination and site OLK7 was used as the reference site, since metals were present at background levels (Table 1.1). Site OLK1 was located inside the metallurgic dump, and contained no beetles for sampling. Assessment of gradient effects is an ideal method for determining whether trends in organismal parameters exist that are associated with chronic exposure to multiple pollutants. This is especially the case when replication of field sites is not feasible. Gradients minimize the influence of environmental factors compared with distant site approaches (Posthuma and Van Straalen, 1993).

The results of field-based studies will invariably differ from the results of research investigations conducted in the laboratory. Kimball and Levins

Table 1.1. Soil concentrations of heavy metals (+/- SD) along the sampling gradient in Southern Poland.

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

(1985) and Cairns (1986) have questioned the degree to which laboratory testing of individual pollutant impacts on organisms can predict the effects observed in natural environments. The response of organisms to environmental stressors tends to be integrated, encompassing many modulating and interacting aspects of the natural environment (Depledge and Fossi, 1994). Experimental parameters are seldom under control in the field, and relationships between pollutants exposure and biological endpoints tend to be inferred and based on the weight-of-evidence approach (Suter et al., 1994).

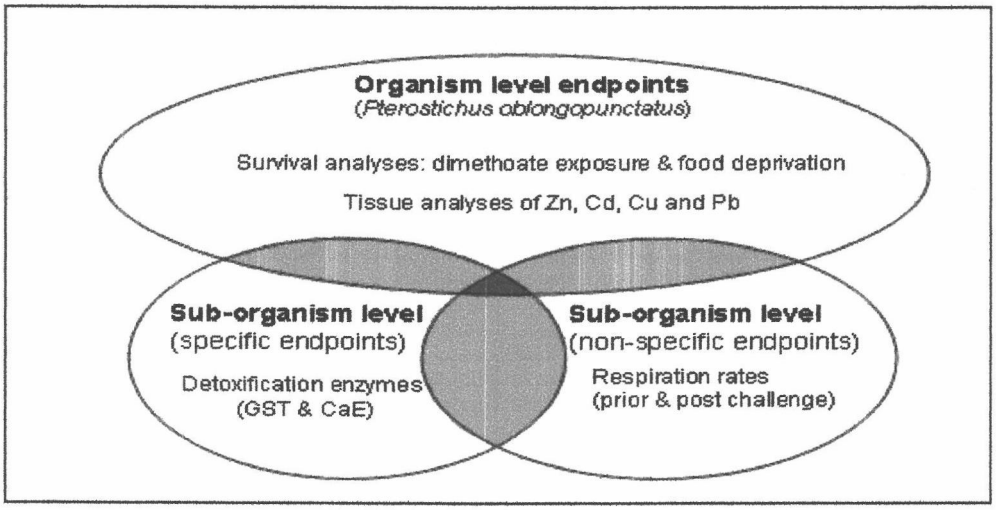
Apart from the confounding factors inherent within field-based research, another difficult challenge in assessing the effects of multiple stressors is the selection of appropriate endpoints to provide relevant information with respect to the mode of action of the stressors and their impact

on the organism (Breitburg *et al.*, 1998). We initiated our studies by examining the survivorship of beetles collected along the pollutant gradient after supplementary exposure to an acutely-acting pesticide and to starvation as a result of food deprivation. Specifically, we chose to perform time to death (TTD) analyses because of limited availability of test organisms, and unequal sample sizes. The method permitted the inclusion of exposure duration as a test parameter (Newman and McCloskey, 1996). At the physiological level, specific and non-specific biomarkers were evaluated to determine their suitability as endpoints for the detection of sub-lethal stress associated with inhabiting the polluted system. This included analysis of the activity of two detoxification enzymes, glutathione S-transferase and carboxylesterase, and the measurement of respiration rates of beetles collected along the pollution gradient. The detoxification enzymes were selected because they represent ubiquitous defense mechanisms against xenobiotic exposure. Respiration rate represents a broad, non-specific parameter to detect potential alterations in physiology following an environmental challenge.

Research conducted at different levels of biological organization will yield information that contributes to understanding of toxicological mechanisms, physiological impacts and potential impacts on survival and fitness. Events that occur at one level of organization may represent the mechanisms that influence the next higher level of organization (Suter, 1995). Sub-organismal endpoints, including biochemical, histological and

physiological characteristics, have intimate links to the organism scale. Figure 1.1 summarizes the scope of this project, incorporating the biomarkers examined at the organism and physiological levels, with which we examined potential costs from exposure to chronic pollution.

Figure1.1. The relationship between organism and sub-organism endpoints in *Pterostichus oblongopunctatus* sampled along the gradient of heavy metal pollution. Shaded areas indicate zones of potential overlap.



Costs of pollutant exposure:

Considerable evidence has demonstrated that inhabiting chronically polluted environments is costly to terrestrial organisms (Strojan, 1978; Bengtsson and Rundgren, 1984; Alstad et al., 1982), including costs measured at the metabolic level (Calow, 1991, Berenbaum and Zangerl, 1994). A central premise to this theory is that organisms have limited energy budgets

that constrain energetic output to production (biomass and reproduction) and support for basic life functions (frequently classified as respiration).

Organisms in anthropogenically stressed environments may therefore, exhibit reduced fitness compared with organisms that inhabit more natural environments. They may also have less capacity to survive supplementary stressors because they are already under a higher degree of energetic constraint. Examples of this phenomenon include elevated low temperature sensitivity in the grass, *Lolium perenne,* after exposure to sulfur-dioxide (Davison and Bailey, 1982) and higher susceptibility to zinc in the snail, *Lymnaea stagnalis,* when parasitized (Guth et al., 1977). Holmstrup (1997) observed that collembola, *Folsomia candida,* had decreased drought survival after previous exposure to copper and nonylphenol.

In addition to the complexities of selecting appropriate endpoints to measure costs, considerations of physiochemical properties of xenobiotics and their potential for mixture effects must be addressed. In metal polluted environments, complex interactions between metals may affect species diversity, abundance, toxicity and bioavailability of other metals. Information regarding the effects of exposure to multiple metals is however, lacking (Hagvar and Abrahamsen, 1990). For instance, cadmium accumulation tended to decrease in the presence of zinc for silkworm larvae (Matsubara et al., 1982) but increased in crickets fed cadmium and lead (Migula et al., 1989). Lindqvist and Block (1998) observed that cadmium and zinc accumulation

rates tended to be linked in female beetles and not males. In addition, the water-soluble fraction of the pollutant may vary by sampling site, greatly affecting its bioavailability. The interaction between acute short-lived toxicants, such as pesticides, and chronic pollutants on terrestrial communities is even less well documented. Forget et al. (1999) determined that certain metals increased the inhibitory effects of insecticides on the copepod, *Tigriopus brevicornis*, demonstrating the need to investigate chronic and acute xenobiotics in combination.

Biomarkers at the organism level:

Experiments on individual beetles were conducted to test the hypothesis that terrestrial invertebrates inhabiting the most polluted sites in a gradient are more susceptible to additional stressors compared with their counterparts in less stressed environments. This was addressed utilizing time to death (TTD) survival analyses on *P. oblongopunctatus* collected along the gradient of heavy metal pollution. Beetles from reference and polluted sites were exposed to topical applications of an organophosphate insecticide, dimethoate, and subjected to food deprivation. Exposure to dimethoate represents an acute-acting and severe challenge to the beetle, while food deprivation mimics a natural and slower acting stressor. Shorter TTD measurements in organisms from more polluted habitats would imply greater

susceptibility to the stressing agent, consistent with the costs of pollution hypothesis.

Metal body burdens were evaluated in individual beetles collected along the sample gradient. Tissue concentrations provide a quantitative assessment of pollutant burdens that may be associated with differential survivorship and trends in physiological response. Furthermore, the accumulation or regulation of tissue concentrations for heavy metals may be assessed.

Certain population scaled endpoints can be predicted from individual level parameters. Prior to our studies on *P. oblongopunctatus*, we compared two population growth models in the pea aphid, *Acrythrosiphon pisum* (Appendix A). Measures that factor in fecundity, growth rate and age structured effects were analyzed in *A. pisum* exposed to an insecticide and cadmium in combination. The results of multiple pollutant exposure included unexpected mixture effects on the fitness of exposed aphids and encouraged us to investigate other heterogeneously polluted sites, such as the metal gradient in Poland (Appendix A).

Biomarkers at the physiological level:

The focus of the analysis of physiological endpoints was to examine whether trends in sub-organism biomarkers can be detected in *P. oblongopunctatus*, in conjunction with chronic exposure to a gradient of heavy

metal environmental concentrations. The establishment of reliable physiological endpoints, often referred to as biomarkers, are especially important in field-based investigations of pollutant impacts, since they have the potential to detect stress before ecological effects develop (Zachariassen et al, 1991). Optimal biomarkers will have the following characteristics: good signal-to-noise ratios, short response time, specificity of response, quantifiable responses, and ease and economy of measurement (Mineau, 1998).

There has been recent emphasis on developing and validation of enzyme activity as a biomarker in ecotoxicology (Mitton, 1997). In general, negative impacts on individual fitness (i.e. costs) may occur if pollutants cause physiological responses to deviate beyond normal ranges (Calow, 1991; Calow and Forbes, 1998). Xenobiotics may completely inhibit enzyme activity, potentially reducing fitness, or, alternatively, the induction of an enzyme above normal levels may be costly because of the high energetic demands that are made (Sibly and Calow, 1989).

The enzyme activity of *P. oblongopunctatus* was measured for non-specific carboxylesterases (CaE) and glutathione S-transferases (GST). CaEs are Phase I enzymes that react with non-polar compounds through hydrolysis, resulting in more polar metabolites, which are 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).

The second physiological biomarker examined was resting respiration rate, measured as the expiration of CO_2. Respiration rates typify a broad ranging, non-specific response to environmental and physiological conditions. It has been argued that nonspecific biomarkers offer a better assessment of the effect of multiple stressors acting simultaneously on an individual (Mineau, 1998). Respiration rates were calculated in individuals collected from three of the metal gradient sites: the two most polluted sites (OLK2 and OLK3) and the reference site (OLK7). In addition to measuring the rate of CO_2 expiration, *P. oblongopunctatus* were exposed to dimethoate, to challenge them with an acute stressor. Pre and post challenge respiration rates were compared to determine if inhabiting chronically polluted sites would affect the ability of beetles to induce resting respiration rates after exposure to acute stress.

During the experiments on enzyme activity and respiration rate, beetles were collected at similar sample sites to those used in the TTD studies to determine if trends could be established between metal exposure, differential survivorship and physiological biomarkers. Furthermore, specific information regarding the influence of sex on physiological response was analyzed to determine the general suitability of these biomarkers for determining the impact of exposure to multiple stressors.

Statement of purpose:

Widdows and Donkin (1991) have proposed a framework to link exposure to pollutant(s) and a biological effect. The framework includes determining tissue concentrations of the pollutant in question, determining a cause-effect relationship and investigating a mechanism for the effect. During our investigations in Southern Poland, we attempted to meet all three criteria using the ground beetle, *P. oblongopunctatus*. Initially, we examined whether differential survivorship could be detected after exposure to stress in ground beetles inhabiting a gradient of pollution. Secondly, we quantified the tissue concentrations of metals in individual beetles. Finally, we investigated sub-organismal biomarkers as indicators for the mechanisms that may influence the organism scale by measuring the trends in two detoxification enzymes and in respiration rate. Integration of these measures of sub-organismal and organismal level impacts provided the basis for addressing the central question: are the ground beetles collected from polluted sites incurring costs as a result of anthropogenic activity compared with their counterparts in less polluted sites?

Chapter 2

Response to Multiple Stressors in Terrestrial Beetles along a Gradient of Heavy Metal Pollution

David Stone, Paul Jepson, Paulina Kramarz, and Ryszard Laskowski

Published by *Environmental Pollution*
Elsevier Science, Oxford, U.K.
Vol. 113 (2001), 239-244

Abstract:

We investigated the responses of invertebrates inhabiting polluted environments to multiple stressors. Carabid beetles (*Pterostichus oblongopunctatus* F.) were subjected to food deprivation and insecticide treatment (dimethoate) to resolve trends associated with a gradient of heavy metal pollution. Metal concentrations along the gradient of five sites ranged from approximately 150 mg/kg to 10500 mg/kg zinc, 136 to 2600 mg/kg lead, and 0.84 to 81.9 mg/kg cadmium. There was no difference in body mass along the pollution gradient. However, the beetles originating from the most contaminated sites were significantly less tolerant to food deprivation than beetles from the reference site. Median survival time was 120 hours for the two most polluted sites, compared with 168 hours at the reference site. Beetles from the two most polluted sites were also significantly more susceptible to dimethoate at 0.1 µg a.i./beetle. Median survival times were 12 and 123 hours for beetles from the two most polluted sites and 359 hours for the reference site. Carabid beetles exposed to chronic pollution therefore, exhibit elevated susceptibility to additional stressors.

Introduction:

One of the major assumptions of ecotoxicology and stress ecology is that there are energetic costs associated with survival and persistence in chronically polluted environments (Hoffman and Parsons, 1989; Sibly and Calow, 1989). This assumption is based upon the premise that organisms must exist under the constraints of a limited energy budget. Significant outputs of energy within this budget are channeled to production (biomass and reproduction) and support for basic life functions (frequently classified as respiration). If survival in a chronically polluted environment requires supplementary detoxification, then there is evidence that these physiological responses are metabolically costly (Calow, 1991).

The diversion of energy to detoxification mechanisms does not necessarily lead to deleterious effects in all situations. Organisms may however, be more subject to reduced fitness after exposure to additional stressors. Examples of this phenomenon include elevated low temperature sensitivity in the grass, *Lolium perenne,* after exposure to sulfur-dioxide (Davison and Bailey, 1982), increased heat sensitivity in five plant species that were tolerant to triazine (Ducruet and Lemoine, 1990) and higher susceptibility to zinc in the snail, *Lymnaea stagnalis,* when parasitized (Guth et al., 1977). The purpose of this study is to test the hypothesis that terrestrial invertebrates inhabiting chronically polluted environments are more susceptible to additional environmental stressors than their counterparts in less stressed environments.

The chronic contaminants under investigation were a suite of heavy metals emitted from zinc and lead smelting industries. The evolution of tolerance in plants to heavy metals has been characterized as one of the best examples of natural selection in action (Bradshaw and McNeilley, 1990). Terrestrial invertebrates may acquire heavy metals from the leaf litter and soil through dermal absorption, ingestion of soil and feeding on contaminated prey. Species that lack high dispersal ability, relative to the scale of pollution, and which may be confined to local zones of pollution for multiple generations, may be exposed to sustained selection pressure. It is also probable that these organisms will encounter additional stressors and that the response to these will be mediated by the history of exposure to other pollutants.

The forest dwelling carabid, *Pterostichus oblongopunctatus* F. (Coleoptera: Carabidae), was collected at sampling sites along a gradient of metal pollution. Carabids are characterized as poor accumulators of heavy metals (Kramarz, 1999), which may result from elevated mechanisms of detoxification and excretion. Through the processes of protein synthesis and catabolism, both detoxification and excretion can be energetically costly (Hawkins, 1991). To address the cost of inhabiting chronically polluted environments, we tested the responses of individual *P. oblongopunctatus* inhabiting sampling sites along a gradient of heavy metal pollution, to the organophosphate insecticide dimethoate and to food deprivation. These supplementary stressors were selected because ground beetles in many polluted

regions may be exposed to pesticide sprays in agricultural systems and they may also endure seasonal reduction in the availability of prey.

Materials and Methods:

Study sites:

All study sites were located along a gradient of heavy metal pollution in the vicinity of Olkusz in southern Poland (approximately 50 17'N/19 31'E to 50 32'N/19 39'E). Mining activity in this region dates back to the medieval period, although intensive industry began in 1967, when the largest Polish zinc smelter was constructed. Currently, there are several mines and two smelters located approximately 4 km apart (consuming about 3 million tons of zinc-lead ore annually). The present dust emission from these smelters to the atmosphere reaches ca. 45 tons per year, with a peak emission of 1140 tons having occurred in 1969. In the late 1980s, the yearly dust precipitation in the region was approximately 118 tons/km^2. This translates to an annual deposition of more than 1000 kg zinc, nearly 200 kg lead, 10 kg cadmium and 31 kg copper per km^2 (Kapeja et al., 1990). As a result, intense soil pollution extends over an area of over thirty square kilometers. Preliminary studies identified 7 sites labelled OLK1 through OLK 7, located along a gradient of heavy metal pollution (OLK1 located nearest the smelter). From these, five sites (OLK2, OLK3, OLK4, OLK6 and OLK7) were selected for further

research based upon concentrations of metals in the humus layer. These sites represent a broad range of pollution, ranging from 150 to 10,500 mg kg^{-1} zinc in dry humus, 140-2600 mg kg^{-1} lead, 0.8-100 mg kg^{-1} cadmium and 11-74 mg kg^{-1} copper (Table 2.1). All sites were dominated by Scots pine (*Pinus sylvestris*) forest with a small number of other tree species (*Quercus sp., Betula sp.*). The soils throughout the gradient are characterized by acidic, podsolized, sandy types with a well developed mor humus layer.

Table 2.1. Mean concentrations of major pollutants (+/- SD) in the humus layer along the heavy metal gradient.

Site	Km*	Zn (mg kg^{-1})		Cd (mg kg^{-1})		Cu (mg kg^{-1})		Pb (mg kg^{-1})	
		MEAN	SD	MEAN	SD	MEAN	SD	MEAN	SD
OLK 2 **	3.5	10454	2618	81.9	17.2	46.9	4.56	2635	120
OLK 3	2.5	5104	729	51.1	19.3	37.6	3.72	1832	215
OLK 4	3.9	1522	135	18.1	2.6	25.6	2.16	870	36.3
OLK 6	7.9	244	78.2	3.3	1.03	15.4	2.68	355	31
OLK 7	31.9	151	34.5	0.84	0.39	10.7	0.96	136	8.8

* Distance from the larger of the two smelters is given.
** OLK2 is actually located between the two smelters, hence the contamination is higher than at OLK3.

Experimental organisms:

Beetles in the sampling sites were collected with pitfall (Barber-type) traps in the form of plastic cups (approximately 200 ml capacity). At each site, 50 traps were distributed along two transects of 25 cups each. Both transects and traps were set at 3 meter intervals and were emptied every fourth day. Preliminary studies showed that a species of ground beetle, *Pterostichus oblongopunctatus* F., was collected in sufficient numbers at each site for use in experiments. Consequently, all comparisons were made with this species. The beetles were transported in plastic boxes with perforated lids containing soil from collection sites, to the laboratory within hours of collection. At 48 hours post-collection, the beetles were used in the experiments outlined below. Beetle mortality before experimentation was very low.

Experimental design:

In the first two experiments, *P. oblongopunctatus* from each site were subjected to known doses of insecticide and in the third experiment beetles were deprived of food, to determine differences in susceptibility to these stressors among sampling sites. Limited numbers of organisms were collected on each sampling occasion and the experiments were run independently. The number of replicates (each consisting of an individual beetle from each site subjected to a single dose) differed depending on the number of available specimens (Table 2.2).

Initially, a range-finding study was conducted to determine the susceptibility of *P. oblongopunctatus* to the organophosphate insecticide dimethoate. The aim of this range- finder was to select a dose that gave a long

Table 2.2 Body mass of *P. oblongopunctatus* used in the dimethoate treatments and food deprivation studies, separated into sex (significant difference between sexes, $p<0.0001$; body mass in low dose experiment (0.1 µg a.i./µl) significantly lower, $p<0.001$).

Experiment	Sex	Average body mass (g)	Standard deviation body mass (g)	Sample size
Low dose (0.1 µg a.i./µl)	Males	0.0517	0.00656	70
	Females	0.0616	0.00839	86
High dose (0.5 µg a.i./µl)	Males	0.0505	0.00628	85
	Females	0.0578	0.00784	99
Food deprivation	Males	0.520	0.00516	36
	Females	0.0609	0.00730	37

enough survival time among beetles for differences in susceptibility to be resolved. Doses of 0.5 and 0.1 µg a.i./beetle gave adequate results and were used for further study (high and low dose exposures, respectively). The beetles were dosed topically using a Hamilton gas-tight syringe. One microliter drops were applied along the suture line between the elytra and the pronotum. The dimethoate was a commercially formulated product with 400 g/l of active ingredient, diluted in distilled water to provide the required dose in 1 µl of

solution. Control individuals were dosed with 1 µl of distilled water. Following dosing, the beetles were observed constantly for two hours. Survival was then monitored at 3, 6, 12, 24, 36, and 48 hours and at 24-hour intervals thereafter. The food deprivation study was conducted in a similar manner, without the application of pesticides. All beetles were weighed to the nearest 0.0001 g on an electronic balance (Precisa, Switzerland) immediately prior to dosing.

All experiments took place in a controlled-temperature room at 20^0 C, >80% relative humidity and a light:dark regime of 16:8 h. The beetles were isolated within individual, transparent boxes with uniformly perforated lids. These boxes were placed into large, plastic containers with wet filter paper at the bottom and partly covered with a sheet of Plexiglass to retain moisture.

Statistical analysis:

Differences in the mass of beetles between site, sex and amongst experiments were analyzed by multi-factor analysis of variance. For each significant factor a Bonferroni test was run to separate means. Although females differed significantly from males in mass, both sexes were combined for survival time analysis because of the low number of individuals from some sample sites. Approximately equal sex ratios were used in all tests.

The recorded times to death (TTD) were analyzed and compared among sites with survival analysis (CSS Statistica and Statgraphics software)

for each pesticide dose and for the food deprivation study (Newman, 1996). The survival analysis was also used to check for differences in susceptibility to dimethoate that might result from variation in the time of sample collection. For this purpose, only data from untreated beetles from the reference site (OLK7) were used. Comparisons of survival curves among populations or sampling times were conducted with a log-rank test (Mantel, 1966). If the tests conducted on all sites detected significance at $p<0.1$, individuals from each population were compared against the control site. For each treatment, the median life expectancy after treatment was calculated.

Results:

The average weight of females was 0.0600 g and the average weight of males was 0.0514 g. Statistically significant differences in body mass were not detected along the pollution gradient ($p>0.3$, Table 2.2). The average masses of beetles were 0.0567 g, 0.0539 g and 0.0564 g in the high dose, low dose, and food deprivation experiments, respectively (Table 2.2). *P. oblongopunctatus* collected during the low dose experiment were significantly lighter than those from the high dose or food deprivation study ($p<0.001$). In all experiments, females weighed more than males ($p<0.0001$; interaction non-significant).

In general, the later the date of sample collection, the shorter the survival time of control beetles. The median survival time of control beetles from OLK7 was 628 hours in the first experiment (low dose), 383 hours in the

second experiment (high dose) and only 167 hours in the food deprivation study.

In the high dose experiment (0.5 μg a.i./μl), the difference in TTD amongst sites, after dimethoate treatment, was significant at p=0.073 (Table 2.3, Fig. 2.1). A pair-wise comparison revealed that only populations from the most polluted sites, OLK2 and OLK3, differed significantly from OLK7 (p=0.043 and p=0.034, respectively). For OLK4 and OLK6, the significance levels were p=0.15 and 0.59, respectively. The median survival time was particularly low for beetles from OLK2; 3.75 hours compared with 13 hours at OLK4, OLK6 and OLK7 (Table 2.3). No beetles from OLK2 or OLK3 survived longer than approximately 48 hours after dosing with dimethoate. In comparison, some individuals from the reference site (OLK 7) survived for greater than one month (Fig. 2.1).

In the low dose experiment (0.1 μg a.i./μl), significant effects in TTD among all sites were detected ($p<0.0001$). This dose offered the best resolution of TTD between populations. Beetles from OLK2 had a median survival time of 12 hours and the difference was highly significant in comparison with the reference site ($p<0.001$). OLK3 also differed significantly from the reference site ($p<0.005$) with a median survival time of 123 hours. The median TTD for OLK6 was 288 hours. OLK4 had the longest TTD with 432 hours. Neither OLK4 nor OLK6 were significantly different from the reference site TTD of 359 hours (Table 2.3, Fig.2.2).

Figure 2.1. Time to death analysis of *P. oblongopunctatus* exposed to 0.5 µg a.i/beetle, among sample sites.

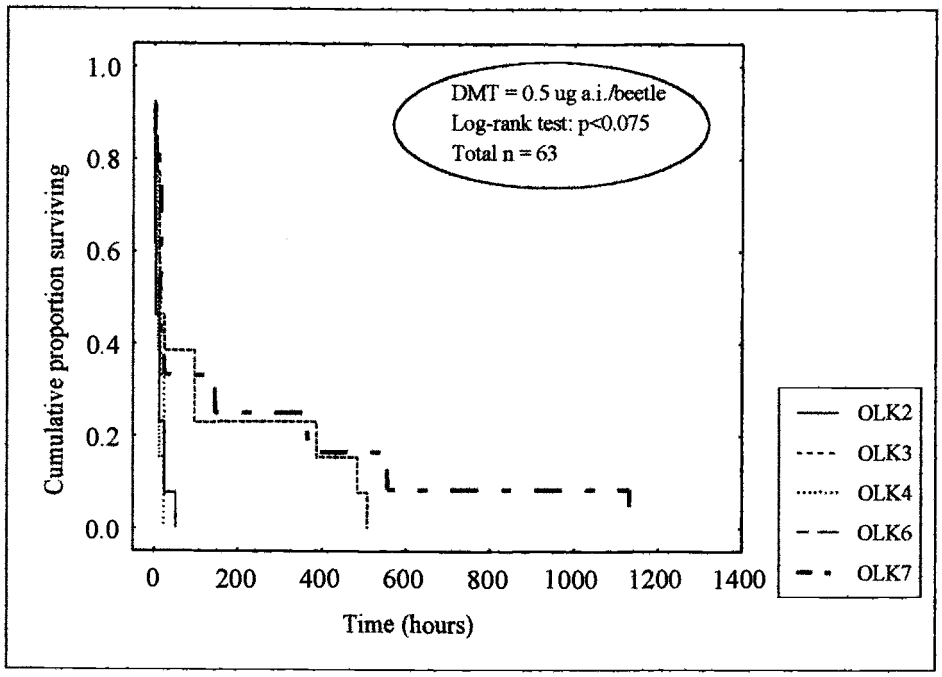


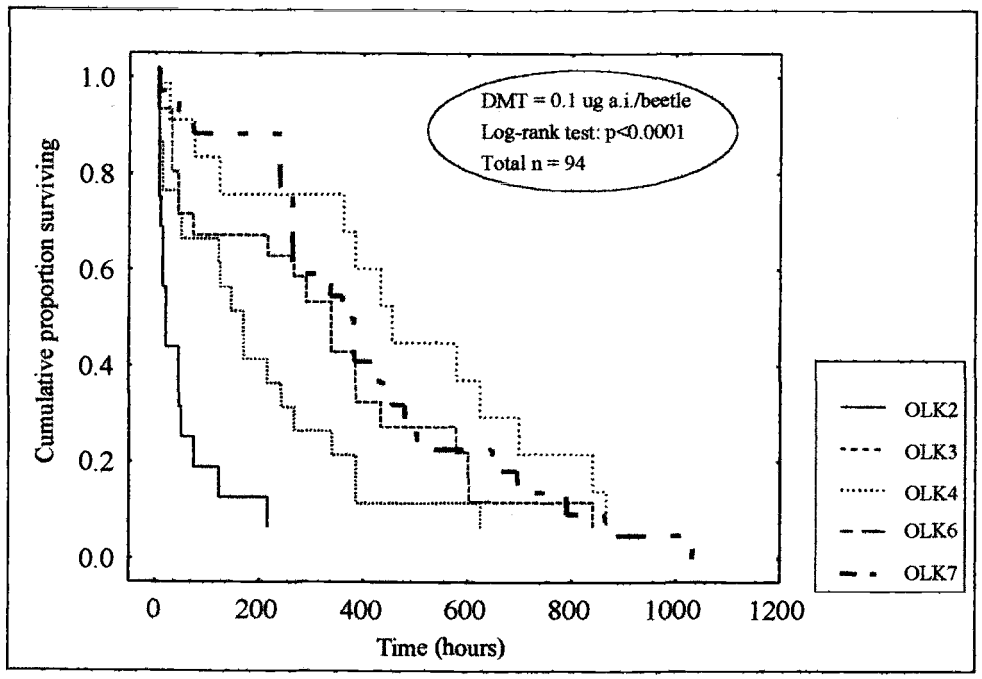
Table 2.3. Median survival times for *P. oblongopunctatus* from study sites in the low and high dose dimethoate treatments and food deprivation study.

Site	**Median survival time in hours (number of beetles tested)**			25-75 percentiles		
	low dose	high dose	starvation	low dose	high dose	starvation
OLK2	12.3* (16)	3.75* (13)	120*	5-43.0	1.5-13	98-144
OLK3	123.3* (20)	10.0* (13)	120*	12-240	3.5-13	98-192
OLK4	431.7 (13)	13.0 (12)	144	120-623	5-25	120-192
OLK6	287.9 (23)	13.0 (13)	167	29-431	13-97	98-216
OLK7	359.1 (22)	13.0 (12)	167	264-503	10-145	144-384

* Indicates survival times that are significantly different than OLK7 ($p<0.1$).

The food deprivation study resulted in differences in TTD significant at $p<0.085$ among all sites. Pairwise comparisons revealed that populations from the two most polluted sites, OLK2 and OLK3, differed significantly from the reference site ($p<0.007$ and $p<0.052$, respectively). The median survival time

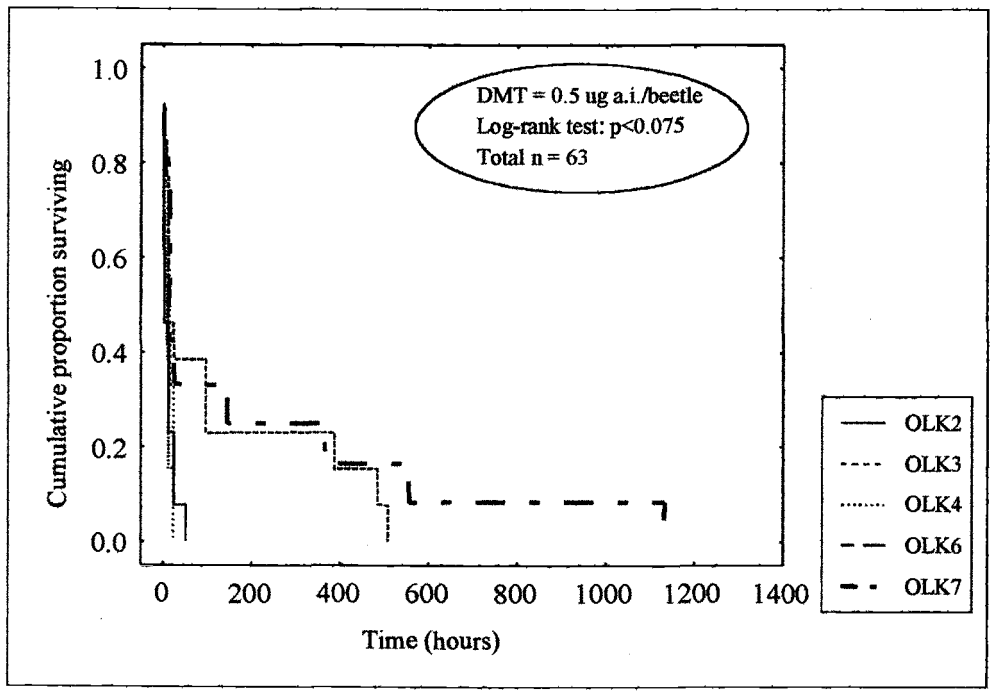
Figure 2.2. Time to death analysis of *P. oblongopunctatus* exposed to 0.1 µg a.i/beetle, among sample sites.



was the shortest in populations from OLK2 and OLK3 (120 hours) compared with 144 hours at OLK4 and 168 hours at OLK6 and OLK7 (Table 2.3, Fig. 2.3).

The estimated median life expectancy demonstrated a clear decreasing trend with increasing pollution level at a dose of 0.1 µg a.i./µl (from 346 hours in OLK7 to 19 hours in OLK2) and in the food deprivation study (from 161 hours in OLK7 to 129 hours at OLK2). These results are summarized in Table 2.4.

Figure 2.3 Time to death analysis of *P. oblongopunctatus* among study sites, in the food deprivation study.



Discussion:

The ability to predict and understand the effects of multiple stressors on the biota has been described as one of the most important challenges facing

Table 2.4. Median life expectancy since the start of the experiment for *P. oblongopunctatus* among study sites in the low and high dose dimethoate treatments and food deprivation study.

Site	Median Life Expectancy in hours (+/- SE)		
	low dose (0.1 µg a.i./µl)	high dose (0.5 µg a.i./µl)	food deprivation
OLK2	19.2 (4.8)	11.1 (3.1)	129.3 (17.7)
OLK3	133.3 (29.2)	9.8 (2.7)	139.5 (17.1)
OLK4	355.9 (54.4)	14.0 (3.5)	140.1 (22.6)
OLK6	254.0 (57.1)	22.3 (6.2)	158.2 (26.5)
OLK7	346.8 (31.0)	36.0 (10.4)	161.4 (13.0)

scientists (Breitburg *et al.*, 1998). There is a potential for any individual stressor to alter a system such that additional stressors have greater impacts when exposure occurs in stressed environments compared with unstressed environments. In this study, the chronic exposure of *P. oblongopunctatus* to high concentrations of heavy metals was shown to increase their susceptibility to additional and realistic stressors.

Research elucidating the mechanism and response of organisms to multiple stressors with dissimilar modes of action is lacking. Hoffman and Parsons (1989) predicted that genetic correlations between tolerance to different environmental stresses tend to be positive. This was based on

research conducted with artificially-selected lines of *Drosohila* sp. comparing tolerance to starvation and to toxic concentrations of ethanol. These predictions do not necessarily apply to all cases of stress tolerance, especially those associated with chemicals with dissimilar modes of action such as metals or pesticides (Hoffman and Parsons, 1989). This research is among a limited number of studies to collect organisms from heavy metal polluted systems and analyze their response to additional stressors.

There is evidence for a range of possible trends in susceptibility to supplementary stressors, in laboratory testing. Forget *et al.* (1999) found synergistic effects in lethality for copepods exposed to mixtures of metals (arsenic, copper and cadmium) combined with insecticides (carbofuran, dichlorvos and malathion). They determined that exposure to metals at sub-lethal concentrations enhanced the inhibitory effects of certain organophosphate and carbamate insecticides towards acetylcholinesterase. Similar to this study, elevated susceptibility to insecticides in combination with metal exposure would not have been detected in traditional, single-chemical bioassays. Both studies lend emphasis to the importance of taking into account multiple pollutants that may be inherent in the system under investigation.

Metals are highly persistent and exert a strong selection pressure over multiple generations to organisms with low-dispersal abilities. In addition, species that are repeatedly exposed to pesticides over several generations may become resistant, although this is rare in predatory invertebrates (Tabashnik

and Johnson, 1999). Several studies have focused on the effects of chronic pollutant exposure on life history traits. Halliday (1990) found that malathion tolerant moths had reduced reproductive performance compared to non-tolerant organisms. Read *et al.* (1987) determined life history changes (absence of summer diapause) in the ground beetle, *Nebria brevicollis*, at a site with high heavy metal concentrations. Kramarz and Laskowski (1999) found a significant decrease in the number of eggs laid by the carabid beetle, *Poecilus cupreus*, after zinc treatment. The spatio-temporal dynamics of these xenobiotics and their interaction with species and environments is crucial to predicting the probability of population persisting or becoming locally extinct (Jepson and Sherratt, 1996, Sherratt and Jepson, 1993).

The beetles inhabiting the most polluted sites (OLK2 and OLK3) were able to maintain a body mass comparable to beetles collected in less polluted sites. This would indicate that they were ingesting contaminated prey in sufficient numbers to maintain body weight, even though some of the prey must have been contaminated. This finding discounts the conclusion that differences in susceptibility could simply be attributed to differences in dose/mass beetle across the gradient. Instead, one possibility is that the beetles residing in OLK2 and OLK3 have incurred physiological or genetic costs, rendering them more susceptible to natural and anthropogenic stress. Possible costs may have arisen through enzymatic induction, altered excretion efficiency mechanisms, enhanced transcription of metal binding proteins or

some other function. Whether the cost is a result of an acclimation (physiological) or adaptive (genetic) process remains unknown.

During the course of this study, beetles had a decreased median survival rate as the season progressed. There was a longer median survival time among untreated organisms collected in early May (used as controls during the dimethoate exposure study) versus those collected in late June (food deprivation study). A likely explanation is that *P. oblongopunctatus* is a spring breeder (Brunsting, 1981), and that the shorter TTD in late summer may occur as a result of changes in lipid composition after egg laying and mating behavior. In addition, some adults that over-wintered successfully may have been in a senescent state towards the end of their lifespans. The difference in temporal susceptibility to stress within the same life stage has obvious implications for the investigation of pollutant impacts if the animals are still laying eggs or contributing to breeding.

Significant differences in TTD for beetles subjected to food deprivation and dimethoate along a pollution gradient indicate that living in a chronically polluted environment has associated costs. In our case, the cost was expressed as an increase in susceptibility of *P. oblongopunctatus* to additional stress in the two most polluted sites. We found a negative correlation between exposure to chronic pollutants and the ability to survive additional stress with a dissimilar mode of action. How this cost influences the evolutionary processes and overall fitness of the population warrants further investigation.

Chapter 3

Trends in Detoxification Enzymes and Heavy Metal Accumulation in Ground Beetles (Coleoptera: Carabidae) Inhabiting a Gradient of Pollution

David Stone, Paul Jepson and Ryszard Laskowski

Submitted to *Comparative Biochemistry and Physiology*
Academic Press,
October 2001, in review

Abstract:

Non-specific 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., 2001a), 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 zinc, 0.174 to 8.66 μg/g lead, and 1.14 to 10.8 μg/g cadmium, whereas copper 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. The role and limitations of detoxification enzymes as suitable physiological biomarkers and accumulation rates of heavy metals are discussed.

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, 2001a). 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 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) was 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., 2001a). The beetles from the two most polluted sites (OLK2 and OLK3) had a significantly lower 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 sex on enzyme expression was also analyzed to determine the suitability of GST and CaE as biomarkers of ecotoxicological stress in Carabidae.

Materials and Methods:

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 about 3 million tons of zinc-lead ore annually). Currently, dust emission from these smelters reaches ca. 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. 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 (approximately 200 ml capacity). Fifty traps were distributed along two transects at each site. Transects and traps were set at 3 meter 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 hours of collection, the beetles were separated by sex and sample location and frozen at –27 ^{0}C.

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 sex and kept in containers for 24 hours to void gut contents. Following this period, the organisms for metal analysis were stored at –70 ^{0}C until analysis for metal content. Whole beetles were dried at 105 ^{0}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.

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 phenyl-thiourea. Homogenization was conducted on ice, using a motor-driven pestle. Immediately after homogenization, beetle guts were centrifuged at 10,000 rpm for 10 minutes. The supernatant was collected (soluble fraction) and stored at –27 ^{0}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) and Grant *et al.* (1989) with modifications. The substrate, 1-chloro-2,4-dinitrobenzene (CDNB), was dissolved in DMSO to give a 75 m*M* concentration of substrate (50 m*M* final concentration). 305 µl of this solution was added to 20 ml of KPO_4 buffer (100 m*M*, 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 m*M* 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 minutes of the reaction at 340 *nm* and 30 ^{0}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 *n*moles 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 m*M*, 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 minutes at a wavelength of 450 *nm* and 30 ^{0}C. Results were corrected for non-enzymatic activity by subtracting blanks (buffer) and converted to units of *n*moles 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 Bradford method (Bradford, 1976), using bovine serum albumin (fraction V) as the standard.

Statistical analyses:

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

Results:

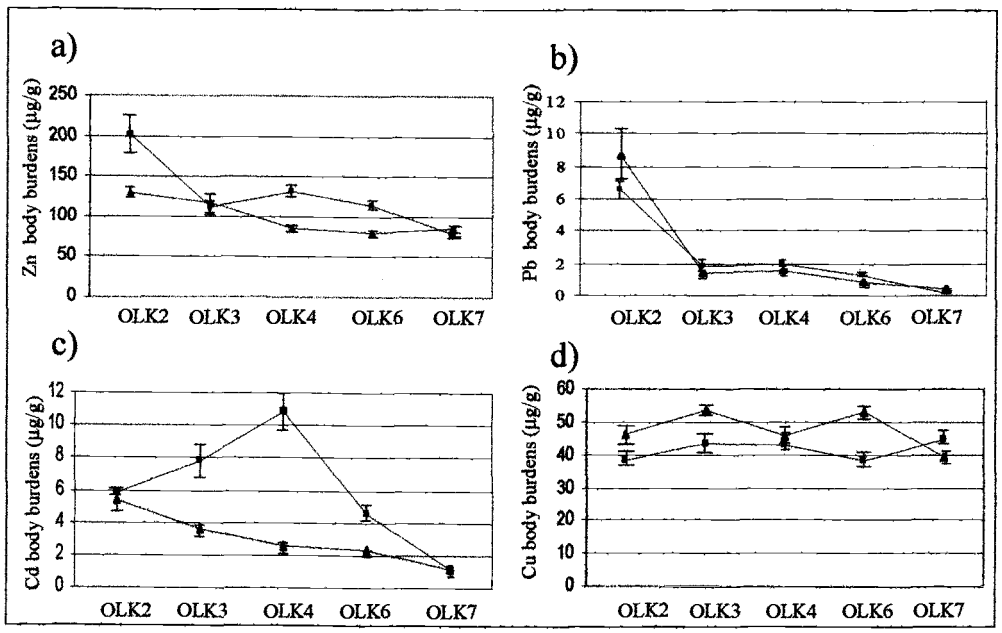
The average concentrations of zinc +/- standard error were 201 (11.8), 112 (23.1), 131 (13.9), 114 (10.6) and 79 (7.6) in female carabids and 130 (12), 118 (14), 86 (5.2), 81 (2.3) and 86 (11.2) in male carabids collected from OLK2, OLK3, OLK4, OLK6 and OLK7, respectively (Fig. 3.1a). Average lead concentrations +/- standard error 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. 3.1b). Average cadmium concentrations +/- standard error 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. 3.1c). Average copper concentrations +/- standard error 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. 3.1d).

For glutathione S-transferase, the average *n*moles of product formed min^{-1} mg $protein^{-1}$ ranged from 150 *n*moles in the reference site to 200.6 *n*moles 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 sex in combination ($p=0.721$). Significant heterogeneity was detected between sites ($p<0.001$), sex ($p<0.001$) and for the interaction between sex and site ($p=0.017$), requiring sex to be tested separately for GST differences.

No significant differences between sites in GST activity among males collected along the sample gradient ($p=0.641$, Figure 3.2). GST activity in females differed significantly between sample sites ($p<0.001$), ranging from 118.4 *n*moles in the reference site (OLK7) to 204.2 *n*moles 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 ($p<0.001$, Figure 3.2). The trend for females was for the enzyme activity to be higher in the more polluted sites.

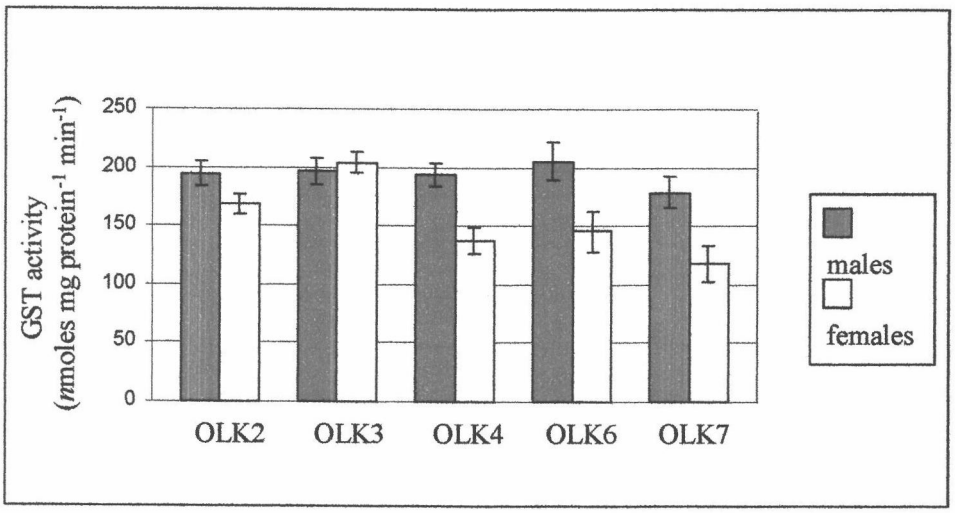
Figure 3.1. Concentrations of a) zinc, b) lead, c) copper and d) cadmium in bodies of *P. oblongopunctatus* along a heavy metal gradient (squares = females, diamonds = males). Error bars represent standard error.



For non-specific carboxylesterases, the average *n*moles of product formed min^{-1} mg $protein^{-1}$ ranged from 71.3 *n*moles 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 sexes in combination, ($p=0.105$). Enzyme activity differed significantly between sites ($p<0.001$) and that the interaction between sex and site was also significant ($p<0.001$). Therefore, sex was analyzed separately for CaE activity.

No significant difference in CaE activity among males between sites (p=0.091, Figure 3.3). CaE activity in females however, differed significantly between sites (p<0.001). Females ranged in CaE activity from 62.7 *n*moles in OLK4 to 103.6 *n*moles 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, Figure 3.3).

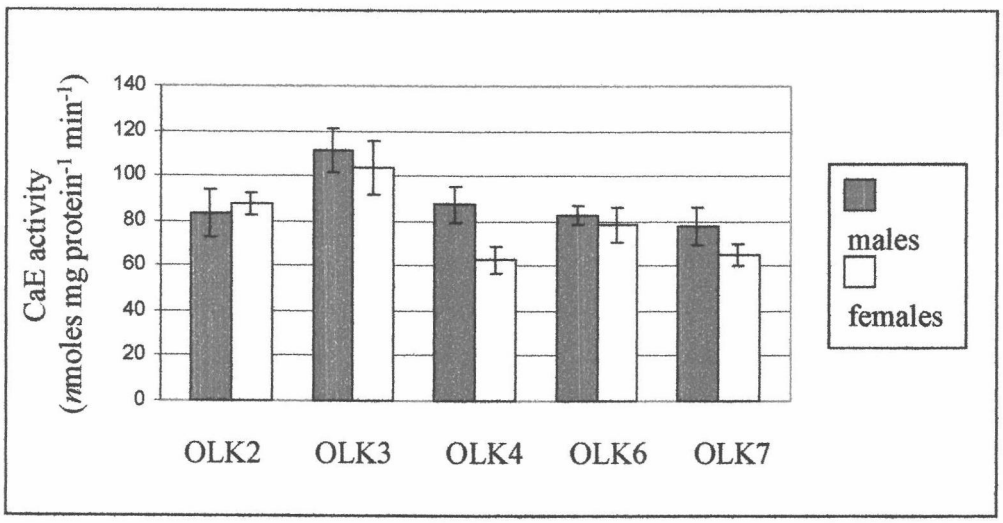
Figure 3.2. GST activity from *P. oblongopunctatus* males and females from sites along the heavy metal gradient. Error bars represent standard error.



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) 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 sex in this study.

Figure 3.3. CaE activity from *P. oblongopunctatus* males and females from sites along the heavy metal gradient. Error bars represent standard error.



Discussion:

Theoretically, glutathione S-transferase (GST) and carboxylesterase (CaE) are ideal biomarkers of ecotoxicological stress. They are ubiquitous

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 spider 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).

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 zinc, lead, cadmium and copper 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 et al. (2001) measured a considerably lower pH at OLK3 compared with OLK2 (5.6 versus 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 sexes 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; Heikens 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. 3.1a). Lead was found in the highest levels at OLK2, but did not accumulate appreciably in relation to concentrations found in the environment (Fig. 3.1b). 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 sexes. 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. 3.1c). 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 uptake becomes detrimental, and these species would no longer be consumed. Lindqvist and Block (1998) observed that cadmium and zinc 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 3.1d). 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 references 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., 2001a), 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.

Chapter 4

Respiration Rate as a Biomarker for Exposure to Chronic Pollution and Acute Stress in Ground Beetles (Coleoptera: Carabidae)

David Stone, Ryszard Laskowski and Paul Jepson

Abstract:

The ground beetle, *Pterostichus oblongopunctatus*, was collected along a gradient of zinc, lead, cadmium and copper pollution in Southern Poland. Resting respiration rate, recorded as the amount of CO_2 produced per individual, was measured to determine if trends could be detected in beetles inhabiting sites with differing metal concentrations. In addition, trends in respiration rate were measured after challenge with the insecticide, dimethoate, to determine the differential response of organisms in conjunction with chronic metal exposure. Male and female beetles collected from the most polluted site (OLK2) exhibited significantly lower resting respiration rates compared with beetles inhabiting an intermediately polluted site and reference site (OLK3 and OLK7, respectively). Following challenge with dimethoate, respiration rates increased significantly in beetles from all sites ($p < 0.001$), suggesting that inhabiting metal polluted sites does not critically impair the ability of *P. oblongopunctatus* to induce respiration rate in response to acute exposure to sub-lethal stress. Disparity in sex was determined with female beetles exhibiting no significant between-site differences in post challenge respiration rates. In males, however, the post-challenge respiration rate remained significantly lower at OLK2 compared with the other sites. Since respiration rate increased markedly after exposure to acute stress, it represents an indirect biomarker to quantify overall physiological response for stressed organisms.

Introduction:

In this study, ground beetles, *Pterostichus oblongopunctatus*, were assayed for trends in respiration rate along a gradient of heavy metal pollution. This is the final component of an ecotoxicological investigation of biomarkers at organism and sub-organism levels for beetles exposed to chronic stress. The initial study conducted on *P. oblongopunctatus* determined a differential survivorship among beetles inhabiting heterogeneously polluted sites along the metal gradient (Stone et al., 2001a). A following study examined trends in two detoxification enzymes to determine their utility as biomarkers of chronic metal exposure (Stone et al., 2001b). The trends in enzyme activity were not distinct, prompting the investigation of respiration rate as a broad ranging endpoint to detect sub-lethal stress. Furthermore, trends in respiratory measurements from stressed and unstressed environments may provide insight into a major assumption of ecotoxicology: survival and persistence in chronically polluted environments is costly (Sibly and Calow, 1989; Hoffman and Parsons, 1989).

Previous studies have provided supporting evidence that inhabiting chronically polluted environments is costly for terrestrial invertebrates (Strojan, 1978; Bengtsson and Rundgren, 1984). A key tenet is that organisms have limited energy budgets for growth, reproduction and respiration. If survival in stressed environments requires supplementary metabolic processes, perhaps trends in respiration rate can be detected and associated with pollutant

exposure. It has been suggested that nonspecific biomarkers, such as respiration rate, are better estimators of multiple stressor effects acting simultaneously on an individual, compared to more specific endpoints (Mineau, 1998). Unfortunately, cause-effect relationships are often inferential when using broad ranging biomarkers, providing potentially conflicting results (Mineau, 1998).

In this study, resting respiration rate was assayed in *Pterostichus oblongopunctatus* collected from two polluted sites (OLK2 and OLK3) and a reference site (OLK7) to determine if a physiological biomarker can be assayed in conjunction with chronic pollutant exposure. An additional aspect of this study was the comparison of respiration rates prior to and after challenge with dimethoate, an organophosphate insecticide. One of the assumptions in stress ecology is that organisms experiencing chronic stress are more susceptible to additional stressors. This assumption was tested by assessing the response of post challenge respiration rates between sites along the gradient. We anticipate that beetles inhabiting the most polluted sites will have a diminished ability to respond to stress, measured as the amount of CO_2 expired in comparison with beetles from the reference site.

Materials and Methods:

The sample site was a heavy metal gradient located near Olkusz, Poland. Several zinc smelters have been emitting pollutants around this site

for decades resulting in soil concentrations ranging from 150 to 10,500 mg kg^{-1} zinc, 140-2600 mg kg^{-1} lead, 0.8-100 mg kg^{-1} cadmium and 11-74 mg kg^{-1} copper in dry humus. Beetles were collected in pit fall traps set three meters apart, along parallel transects.

After collection, beetles were returned to the laboratory and housed in control chambers (15 ^{0}C and approximately 40% RH). Individuals were separated into plastic containers and held overnight, to void stomach contents. Respiration rate was measured with a computer controlled Micro-Oxymax Respirometer (Columbus Instruments, Columbus, OH, USA). This system consists of 30 channels to measure individual respiration rates and was set to record O_2 consumption and CO_2 production every four hours. A Duracell Procell Zinc-Air Medical Battery DA146 (8.4 V) that consumes a known quantity of oxygen per minute was connected to the respirometer for calibration.

Resting respiration rates were calculated over a twenty-four hour period in individual beetles collected from the most polluted site (OLK2), the second most polluted site (OLK3) and the reference site (OLK7) along the heavy metal gradient. Resting respiration rate reflects only the basic metabolic processes to sustain life. Within each chamber, a wetted piece of filter paper was inserted to prevent the beetles from desiccating. Beetles from varying sites were randomly placed into the respiration chamber to minimize any potential "tank effects." If possible, equal numbers of beetles from each site

were placed into the chambers for each experiment. The respiration data was divided into two sets, using the same beetles for each set. The first set measured the respiration rates in beetles for 24 hours prior to topical exposure with dimethoate. The second set measured respiration rates after application of dimethoate in the same beetles, to determine the influence of acute challenge on physiological response. In both sets of data, the first measurement was discarded, since this tended to be an unreliable measurement. To minimize potential effects of circadian activity, the tests were conducted at similar times of the day.

For the second data set, beetles were challenged with dimethoate through topical application between the thorax and abdomen on the dorsal side. Dimethoate is an acute-acting organophosphate insecticide that will challenge the metabolic processes. Dimethoate was formulated with distilled water to 0.25 μg/μl a.i. and administered with a gas-tight Hamilton syringe. After dosing, the beetles were placed in plastic containers to allow the insecticide to completely dry and absorb. Before returning the dosed beetles to their respective chambers, the filter paper was rewetted to prevent desiccation of the test organisms.

Beetles were weighed and their sex determined before each experiment was initiated. For statistical analyses the respiration rates were measured as CO_2 production instead of O_2 consumption because of the better signal-to-noise ratio for CO_2. Final resting respiration data was calculated in μl CO_2

produced $hour^{-1}$ $gram^{-1}$ over a twenty-four hour period, recording data every four hours. The three lowest respiration rates (50% of the recordings) from an individual were pooled for each site and sex to analyze the differences among sites and sexes. Resting respiration rate reflects the basic metabolic processes to sustain life, reducing the contribution of active movement when analyzing the data. Separate analyses were conducted for pre dosing and post dosing experiments. The sample size was nine, fourteen and seventeen males from OLK2, OLK3 and OLK7, respectively. For females, seventeen, fifteen and fifteen beetles were analyzed from OLK2, OLK3 and OLK7 respectively.

Equality of variance was analyzed using Levene's Homogeneity test. A General Linear Model was conducted to examine differences in respiration rates among sites for pre challenge experiments and post challenge experiments, followed by Dunnett's post hoc test if necessary. A paired t-test was used to determine the significance of pre dosing and post dosing respiration rates within each site. Since previous studies indicated differences between male and female carabids in physiological parameters (Stone et al., 2001b), all analyses were conducted on each sex separately. Statistical analyses were run using SPSS version 10.0.

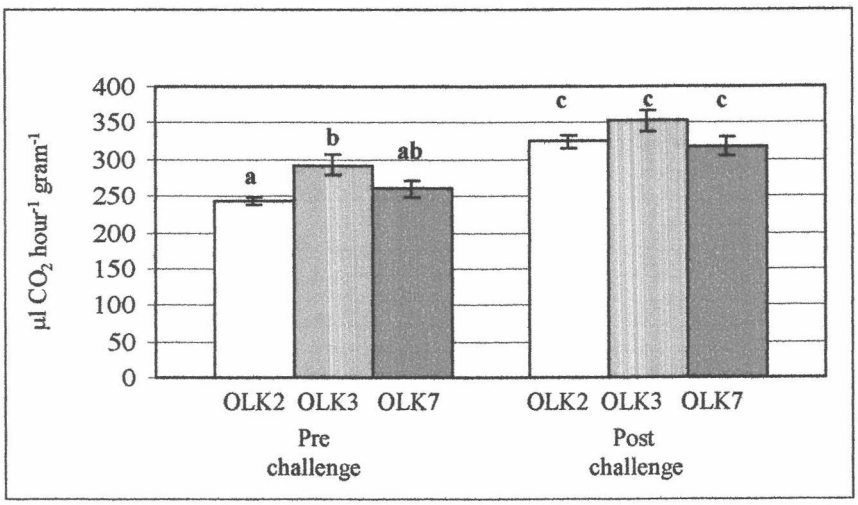
Results:

The mass of female beetles (fresh weight) was 56.7 mg for OLK2, 53.8 mg for OLK3 and 58.4 mg for OLK7. In male beetles, the mass was 54.4 mg

for OLK2, 52 mg for OLK3 and 49 mg for OLK7. No significant differences in mass were detected within sexes (t-test, p=0.05).

The first data set reported is the average resting respiration rate calculated from individual carabids prior to challenge with dimethoate. For females, beetles from OLK3 had the highest respiration rates. Respiration rates (+/- standard error) were 242.5 (5.8), 292.5 (13.9) and 259.5 (12.2) µl CO_2 produced $hour^{-1}$ $gram^{-1}$ from OLK2, OLK3 and OLK7 respectively (Fig. 4.1). Differences were significant between sites (p=0.005), with OLK3 being significantly higher compared with OLK2 (p= 0.005). Respiration rates (+/- SE) for males from OLK2, OLK3 and OLK7 were 194.6 (16), 228.4 (11.9) and 252.2 (9.3) µl CO_2 produced $hour^{-1}$ $gram^{-1}$, respectively (Fig. 4.2). A

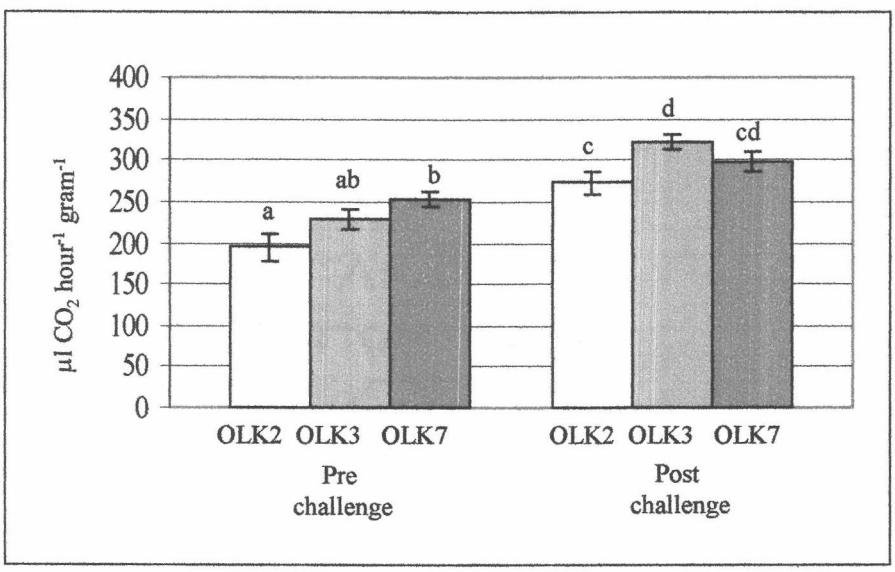
Figure 4.1. Average resting respiration rates for pre and post challenge experiments in *P. oblongopunctatus* females. Different letters denote significant differences ($p \leq 0.05$) and error bars represents standard errors (n= 47 total).



significant difference in respiration rate was determined among sites (p=0.003) with OLK7 having a significantly higher rate compared with OLK2 (p=0.01).

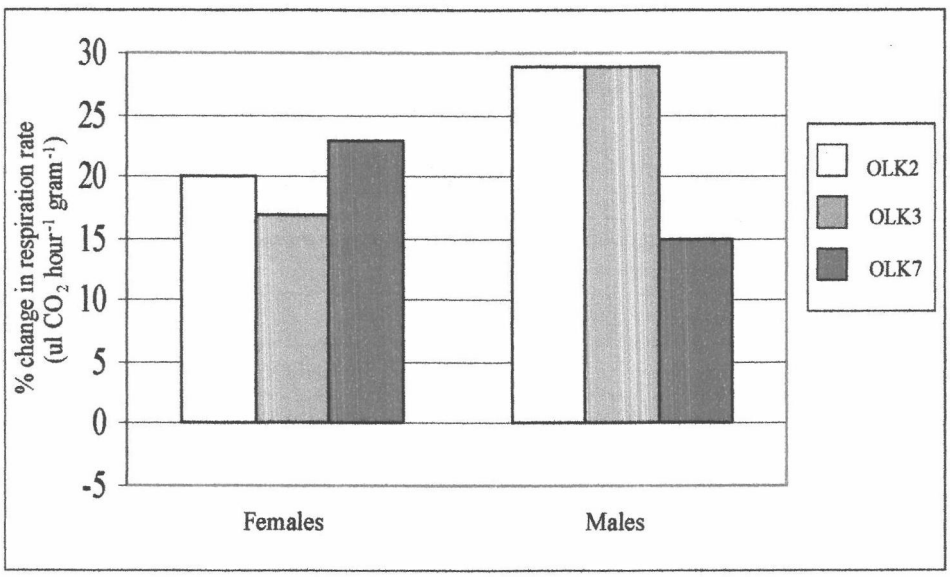
In females, the post challenge respiration rates were highest in beetles collected from OLK3. Resting respiration rates (+/- SE) were 324.1 (10), 352.3 (13.3) and 316 (12.9) µl CO_2 produced $hour^{-1}$ $gram^{-1}$ from OLK2, OLK3 and OLK7, respectively (Fig. 4.1). These rates were not significantly different among all sites (p= 0.093). In males, respiration rates (+/- SE) from OLK2, OLK3 and OLK7 after exposure to dimethoate were 272.7 (14.7), 322.1 (10), and 298.1 (11.4) µl CO_2 produced $hour^{-1}$ $gram^{-1}$, respectively (Fig. 4.2). Significant difference among sites was detected (p= 0.03) with OLK3 having a significantly higher rate than OLK2 (p=0.023).

Figure 4.2. Average resting respiration rates for pre and post challenge experiments in *P. oblongopunctatus* males. Different letters denote significant differences ($p \leq 0.05$) and error bars represent standard error (n= 40 total).



The ratio between the average resting respiration rate pre and post dimethoate challenge was calculated for each sex and site. Females increased their respiration rates by 20%, 17%, and 23% from OLK2, OLK3 and OLK7, respectively (Fig. 4.3). Males increased their respiration rates after exposure to dimethoate by 29%, 29% and 15% from OLK2, OLK3 and OLK7, respectively (Fig. 4.3). At each site and for both sex, respiration rates were significantly higher after exposure to dimethoate compared with rates before pesticide challenge (t-test, $p<0.001$).

Figure 4.3. Percent change in respiration rates following exposure to dimethoate. Zero percent represents the pre-dosing respiration rate.



Discussion:

The majority of studies examining invertebrate respiration rates and heavy metal exposure have focused on aquatic organisms (Spicer and Weber, 1991). Santos et al. (2000) found that oxygen consumption in the decapod, *Farfantepenaeus paulensis*, was reduced at all concentrations of copper and zinc tested. Gaudy et al. (1991) found sub-lethal effects of cadmium on the respiratory metabolism of the crustacean, *Leptomysis lingvura*. Lawrence and Poulter (1996) found significant impairment of respiration rate in the amphipod, *Gammarus deubeni*, by copper. In contrast, Wicklum and Davies (1996) found no effect of Cd on resting and active respiration rates in the predatory leech, *Nephelopsis obscura*. In vertebrate studies, Hopkins et al. (1999) found that banded water snakes (*Nerodia fasciata*) inhabiting coal combustion wastes had elevated standard metabolic rates compared with snakes from reference sites. Similarly, tadpoles (*Rana catesbeiana*) collected from coal-ash ponds revealed 40-97% higher O_2 consumption rates compared with frogs from reference ponds (Rowe et al., 1998) and crayfish (*Procambarus acutus*), exhibited elevated metabolic rates from contaminated versus uncontaminated habitats (Rowe et al., 2001).

It is noteworthy that very few studies have examined the respiratory responses of terrestrial invertebrates after exposure to xenobiotics. In part, this may be the result of inadequate knowledge or interest of respiratory processes in terrestrial invertebrates and pollutant mechanisms. In one study, Knigge and

Köhler (2000) observed increased respiration rates in the isopod, *Porcellio scaber*, as their consumption of lead increased. At the population level, however, there were no significant differences between pre-exposed and non pre-exposed populations of isopods. Ortel (1991) found oxygen consumption rates could be influenced in *Pimpla turionellae* (Ichneumonidae) after exposure to cadmium and lead, due to shifts in lipid metabolism and water production. However, Khalil et al. (1995) did not distinguish any effect of cadmium on respiration rates of *Porcellio scaber*. In experiments on the centipede, *Lithobius mutabilis*, Laskowski et al. (1998) found significant increases in respiration rates of animals one week after exposure to dimethoate, but not after 1, 2, 3 or 14 days post exposure. In copper-treated centipedes, respiration rates decreased 26 and 43 days after treatment, but later recovered to their initial levels (Laskowski et al., 1998).

It is evident from these studies that the response of respiration rate is highly variable depending on pollutant(s) type and organism under investigation. In this study, significantly lower CO_2 expiration rates were detected in male and female carabids from OLK2 prior to dimethoate challenge (Figs. 4.1 and 4.2). Beetles collected from OLK2 had the highest body burdens of zinc and lead (and cadmium for males), signifying that the elevated accumulation of these metals may influence respiration rate. However, whereas males from OLK2 were significantly lower than their counterparts from the reference site, females from the most polluted site were significantly

lower than other females from OLK3 and not the reference site. Beetle mass did not appear to influence respiration rate as no significant differences were detected between sample sites within each sex.

The highest respiration rate occurred in *P. oblongopunctatus* females from OLK3. Similarly, in a previous study, OLK3 females had the highest expression of carboxylesterase and glutathione S-transferase detoxification activities compared with females from other sites along the gradient (Stone et al., 2001b). This observation suggests that enzyme induction may contribute to overall physiological activity, but the contribution is most likely slight (based on the absence of this association in OLK2 females).

Following dimethoate challenge, respiration rates were not significantly different in females between sites, regardless of the body burdens of zinc, lead, cadmium and copper (Fig. 4.1). Possibly, females are highly adept at inducing detoxification enzymes, irrespective of metal accumulation, thus raising overall respiration rate through increased energetic demands. Enzymes such as glutathione S-transferase have been implicated in the detoxification process of organophosphate pesticides (Yu, 1996) and may have been readily induced in female carabids following dimethoate challenge.

For male beetles, differences in respiration rates following dimethoate challenge were detected, with the most contaminated beetles (OLK2) demonstrating a significantly lower respiration rate compared with OLK3 males. Male and female differences pre and post challenge may be the result

of different life history strategies, especially in relation to reproductive processes such as vitellogenesis and potential variations in diet.

Despite the lower respiratory rate measured at OLK2 in the post challenge experiments, males from the two most polluted sites were able to increase their rate of CO_2 expiration by nearly 30% after dimethoate challenge, whereas females averaged a 20% increase in post challenge respiration rates (Fig. 4.3). This increase in respiration rate was highly significant for both sexes ($p<0.001$), dramatically increasing CO_2 production in beetles at all sites. It was obvious from this elevated physiological output that dimethoate was stressful to the beetles. However, no mortality was recorded from any site. This finding suggests that inhabiting metal polluted sites does not critically impair the ability of *P. oblongopunctatus* to induce respiration rate after acute exposure to sub-lethal stress or that the induction observed was lethal at the organism level.

In summary, pre challenge respiration rates were lower in beetles collected from the most polluted site compared with other sites along the gradient. Based on this finding, a broader role for respiration-based biomarkers in stressed environments is encouraged. This study has demonstrated the necessity of considering sex in physiological investigations, emphasized by the differences in male post challenge respiration rates, absent in female carabids. Regardless of the pollutant burden, respiration rates increased noticeably after dimethoate challenge, indicating the utility of

respiration rate as a biomarker to quantify the ability of organisms inhabiting chronically polluted environments to respond to additional stress.

Conclusion

In contrast to the effects of transient pollution, chronic pollution can have lasting impacts because it may alter the environment in which organisms live (Walker et al., 2001). As more consideration is given to chronic, heterogeneously polluted systems, it is imperative to gain a better understanding of the applications and limitations of potential biomarkers to evaluate exposed organisms. In our study, several biomarkers, including detoxification enzymes, respiration rates and time to death survival analyses were assessed in ground beetles, *Pterostichus oblongopunctatus*, along a gradient of heavy metal pollution. Specifically, the endpoints were examined to determine whether beetles inhabiting chronically polluted sites (OLK2 and OLK3) incurred costs compared with beetles at less polluted sites and the reference site (OLK7).

The best indicator of costs associated with inhabiting the most polluted sites was the differential survival times for beetles subjected to additional stressors. The costs were expressed as decreased survivorship in *P. oblongopunctatus* from OLK2 and OLK3 compared with their counterparts in less contaminated sites. A negative association between exposure to chronic pollutants and the ability to survive additional stress with a dissimilar mode of action was detected. In the first experiments, the additional stress was an acute toxicant, dimethoate. The second experiment subjected beetles to food

deprivation, a much slower acting stressor compared with dimethoate exposure.

The beetles inhabiting OLK2 and OLK3 were capable of maintaining a body mass comparable to beetles collected in less polluted sites. This finding discounts any notion that differences in susceptibility can be explained by differences in dose, as a function of beetle mass. Possibly, the beetles residing in OLK2 and OLK3 have endured some degree of physiological impairment, rendering them more susceptible to natural and anthropogenic stress. To address this possibility, we investigated three sub-organism level endpoints.

The initial biomarkers measured were the detoxification enzymes, glutathione S-transferase (GST) and non-specific carboxylesterase (CaE). We observed that female beetles from OLK2 and OLK3 had significantly higher expression of GST compared with females collected from the reference site (OLK7), similar to the finding by Wilczek et al. (1997) for spiders inhabiting metallurgic dumps and ants collected from industrially polluted forests (Migula and Głowacka, 1996). In addition, female beetles from OLK3 had a significantly higher level of CaE activity compared with OLK7. No significant trends in activity were detected in males for either CaE or GST across the sample gradient. Even though the soil concentrations of Zn, Cd, Pb and Cu were highest at OLK2, the highest level of enzyme activity for both sexes occurred at OLK3. This may be due to a higher water soluble fraction of Zn

measured at OLK3 (15.9 mg/kg) compared with OLK2 (8.4 mg/kg; Stefanowicz et al., 2001).

It is unclear why male beetles did not significantly fluctuate in enzyme activity between sites, in contrast to females. Perhaps detoxification enzymes are more inducible in female beetles exposed to physiological stress or females have inherent variations due to differences in reproductive physiology, diet or other factors operating independently from metal exposure. This observation is not unique to *P. oblongopunctatus* as differences in GST and CaE activity among sexes have been reported for other invertebrates (Almar et al., 1987). The difference observed between sexes emphasizes the challenges involved with interpreting physiological data as a function of stress exposure in field-based experiments. Additional complexities include variation in enzyme activity within sample sites and the inability to manipulate several experimental parameters. Accordingly, whether a strong relationship exists between differential survivorship, metal accumulation and enzyme activity is somewhat ambiguous.

The final sub-organism endpoint assessed were trends in respiration rate associated with sites along the pollutant gradient. In part, respiration rate was chosen as a suitable biomarker because of the broad physiological assessment it can provide compared with specific enzyme activity (Mineau, 1998). A notable finding was that male and female beetles collected from OLK2 exhibited significantly lower CO_2 expiration rates compared with OLK7

and OLK3, respectively. The highest accumulation rates of Zn and Pb (and Cd for males) occurred at OLK2 and may indirectly influence respiratory rates through an overall physiological inhibition. This possibility warrants further investigation for the use of respiration rate as a biomarker in chronically polluted systems.

For both sexes, the effect of dimethoate challenge on respiration rate was highly significant ($p<0.001$), causing a dramatic increase in the rate of CO_2 production in beetles from all sites. Even though dimethoate was stressful to the beetles, no mortality was recorded during the experiments. The increase in respiration rate implies that inhabiting metal polluted sites does not critically impair the ability of *P. oblongopunctatus* to induce respiration rate after acute exposure to sub-lethal stress. Based on the significant elevation in respiration rate after acute challenge, this biomarker is encouraged as a tool to assess the ability of an organism exposed to chronic pollution to mount a physiological response against additional stressors.

Even though carabids are characterized as poor accumulators of heavy metals (Kramarz, 1999; Heinkins, 2000), we found distinct trends in some of the metals analyzed (Pb and Zn) and more variable trends in other metals (Cd and Cu). Tissue concentrations of zinc had the highest accumulation at the most polluted site (OLK2), with female beetles having the maximum concentration (201 mg/kg). Beetles from OLK2 contained the highest amounts of lead, but did not accumulate the metal appreciably in relation to

concentrations found in the environment, similar to previous studies (Bengtsson and Rundgren, 1984, Beyer et al. 1985). Cadmium uptake displayed the greatest differences between sexes. Males exhibited an expected pattern of accumulation, with the highest tissue concentrations occurring at the most polluted sites. Females, however, had the most accumulation at OLK4, followed by OLK3 and OLK2. Copper levels fluctuated slightly among the sampling sites with no apparent trends. This suggests that copper uptake is reduced or efficiently regulated at the more polluted sites.

What is the relationship between costs from chronic pollutant exposure and the endpoints measured in this thesis? The finding that Zn (the most prevalent pollutant) was present in the highest tissue concentrations at the most polluted sites provides the confirmation for heterogeneous exposure. From the sites that had the highest tissue concentrations of Zn, we observed that the beetles were significantly more susceptible to dimethoate and food deprivation. This association at organism level encouraged us to investigate several specific and non-specific biomarkers at the sub-organism level.

GST activity was significantly elevated in female beetles, similar to the accumulation of zinc and the decreased survivorship in OLK2 and OLK3. No such relationship was found among male beetles. CaE expression proved a less distinct biomarker, as evident in elevated activity for OLK3 but not OLK2 females. The trends analyzed among GST and CaE did not provide clear

indication that beetles incurred costs as a result of alterations in enzyme activity associated with chronic metal exposure.

Respiration rates were lowest at the most polluted site for both sexes. When challenged with dimethoate, the beetles from all sites were able to significantly increase respiration rates, indicating that from an overall physiological perspective, inhabiting polluted sites was not costly. These findings are intriguing since the sub-organismal level must have intimate links with the organismal level, yet the association between levels measured in this study was not distinct. Clearly, the beetles from the most polluted sites were less able to cope with additional stressors. Future experiments planned for *P. oblongopunctatus* include the quantification of lipid content in male and females along the gradient, analysis of tissue metal concentrations in prey, investigation of life history traits and the use of new biomarkers. It is hoped that future studies, in conjunction with the work presented in this dissertation, will provide a comprehensive understanding of the costs to *Pterostichus oblongopunctatus* as a result of exposure to chronic pollution.

References:

Almar, M.M., Diaz-Mayans, J., and Romero, F.J. (1987). Glutathione content and GSH S-transferase activity in midgut gland of *Procambrus clakrii*. Sex differences, the effect of fasting, and their implications in cadmium toxicity. *Comp. Biochem. Physiol.* **87C**, 433-435.

Alstad, D.N., Edmunds, G.F., and Weinstein, L.H. (1982). Effects of air pollutants on insects populations, *Ann. Rev. Entomol.* **27**, 369.

Bengtsson, G. and Rundgren, S. (1984). Ground-living invertebrates in metal-polluted forest soils. *Ambio* **13**: 29-33.

Berenbaum, M. R. and Zangerl, A.R. (1994). Costs of inducible defense: protein limitation, growth, and detoxification in parsnip webworms. *Ecology* **75**, 2311-2317.

Beyer, W.N.,Patte, O.H., Sileo, L., Hoffman, D.J. and Mulhern, B.M. (1985). Metal contamination in wildlife living near two zinc smelters. *Environ. Pollut.* **38A**, 63-86.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principles of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

Bradshaw, A.D., and McNeilley, T. (1990). The essential qualities. In: Shaw, A.J. (Ed.) *Heavy metal tolerance in plants: Evolutionary aspects* (edited by A.J. Shaw), pp. 323-334, Boca Raton, FL, USA.

Breitburg, D.L., Baxter, J.W., Hatfield, C.A., Howarth, R.W., Jones, C.G., Lovett, G.M and Wigand, C. (1998). Understanding effects of multiple stressors: ideas and challenges. In: Pace, M.L. and Groffman, P.M. (Eds.)

Successes, Limitations and Frontiers in Ecosystem Science. Springer-Verlag, New York, pp.416-431.

Brunsting, A.M.H. (1981). Distribution patterns, life cycle and phenology of *Pterostichus oblongopunctatus* F. (Col., Carabidae) and *Philonthus decorus* Grav. (Col., Staphylinidae). *Netherlands J. of Zool.* **31**, 418-452

Cairns, J. (1986). The case for direct measurements of environmental responses to hazardous materials. *Water Res. Bull.* **22**, 841.

Calabrese, E.J. and Baldwin, L.A. (1999). Chemical hormesis: Its historical foundations as a biological hypothesis. *Toxicol. Pathol.* **27**, 195-216.

Calow, P. (1991). Physiological costs of combating chemical toxicants: ecological implications. *Comp. Biochem. Physiol.* **100C** 3-6.

Calow, P. (1996). Variability: noise or information in ecotoxicology? *Environ. Toxicol. Pharm.* **2**, 121-123.

Calow, P. and Vorbes, V.E. (1998). How do physiological responses to stress translate into ecological and evolutionary processes? *Comp. Biochem. Physiol.* **120A**, 11-16.

Chrascina, M., Kafel A., and Migula, P. (1996). Patterns of detoxifying enzymes in larval stage of *Smerinthus ocellatus* L. exposed to cadmium, tocopherol or quercetin. *Studia Societatis Scientiorum Torunensis* **4**, 31-37.

Culliney, T.W. and Pimentel, D. (1987). Preference of the green peach aphid, *Myzus persicae*, for plants grown in sewage sludges. *Bull. Environ. Contam. Toxicol.* **39**, 257.

Davison, A.W. and Bailey, I.F. (1982). SO_2 pollution reduces the freezing resistance of ryegrass. *Nature* **297**, 400-402.

Deplegde, M.H. and Fossi, M.C. (1994). The role of biomarkers in environmental assessment (2). Invertebrates, *Ecotoxicology* **3**, 161.

Donker, M.H. and Bogert, C.G. (1991). Adaptation to cadmium in three populations of the isopod *Porcellio scaber. Comp. Biochem. Physiol.* **100C**, 143-146.

Ducruet, M.H. and Lemoine, Y. (1985). Increased lead sensitivity of the photosynthetic apparatus in triazine-resistant biotypes from different plant species. *Plant Cell Physiol.* **26**, 419-429.

Eaton, D.L. and Bammler, T.K. (1999). Concise review of the glutathione S-transferases and their significance to toxicology. *Toxicol. Sci.* **49**, 156-164.

Forget, J, Pavillon, J., Beliaeff, B., and Bocquene, G. (1999). Joint action of pollutant combinations (pesticides and metals) on survival (LC50 values) and acetylcholinesterase activity of *Tigriopus brevicornis* (Copepod, Harpacticoida). *Environ. Toxicol. Chem.* **18,** 912-918.

Gaudy, R., Guerin, J.-P., and Kerambrun, P. (1991). Sublethal effects of cadmium on respiratory metabolism, nutrition, excretion and hydrolase activity in Leptomysis lingvura (Crustacea: Mysidacea). *Marine Biol.* **109**, 493-501.

Gomori, G. (1953). Human esterases. *J. Lab. Clin. Med.* **42**: 445-453.

Grant, D.F., Bender, D.M., and Hammock, B. D. (1989). Quantitative kinetic assays for glutathione S-transferase and general esterase in individual mosquitoes using an EIA reader. *Insect Biochem.* **19**, 741-751.

Grant, D.F., Dietze, E.C., and Hammock B.D. (1991). Glutathione S-transferase isoenzymes in *Aedes aegypti*: Purification, characterization and isoenzyme-specific regulation. *Insect Biochem.* **21**, 421-443.

Grelle, C. and Descamps, M. (1998). Heavy metal accumulation by *Eisenia fetida* and its effects on Glutathione S-Transferase activity. *Pedobiologia* **42**, 289-297.

Guth, D.J., Blankespoor, D. and Cairns, J. (1977). Potentiation of zinc stress caused by parasitic infection of snails. *Hydrobiol.* **55**, 225-229.

Habig, W.H., Pabst, M.J., and Jakoby, W.B. (1974). Glutathione S-transferases: The first enzymatic step in mercapturic acid formation. *J. Biol. Chem.* **249**, 7130-7139.

Hagvar, S. and Abrahamsen, G. (1990). Microarthropoda and Enchytraeidae (Oligochaeta) in naturally lead-contaminated soil: a gradient study, *Environ. Entomol.* **19**, 1263.

Halliday,W.R. (1990). Comparative fitness of malathion-resistant and susceptible Indian meal moth (Lepidoptera: Pyralidae). *J. Entomol. Sci.* **25**, 239-245.

Hawkins, A.J.S. (1991). Protein turnover: a functional appraisal. *Funct. Ecol.* **5**, 222.

Heinkins, A., Peijnenburg, W.J.G.M., and Hendriks, A.J. (2000). Bioaccumulation of heavy metals in terrestrial invertebrates. *Environ. Pollut.* **113**, 385-393.

Heliövaara, K. and Väisänen, R. (1993). *Insects and Pollution.* CRC Press, Boca Raton, FL, 124.

Hoffman, A. and Parsons, P. (1989). An integrated approach to environmental stress tolerance and life-history variation: desiccation tolerance in Drosophila. *Biol. J. Linn. Soc.* **37**, 117-136.

Holmstrop, M. (1997). Drought tolerance in *Folsomia candida* Willem (Collembola) after exposure to sublethal concentrations of three soil-polluting chemicals. *Pedobiologia* **41**, 361-368.

Hopkins, W.A., Rowe, C.L., and Congdon, J.D. (1999). Elevated trace element concentrations and standard metabolic rate in banded water snakes (*Nerodia fasciata*) exposed to coal combustion wastes. *Environ. Toxicol. Chem.* **18**, 1258-1263.

Iio, M., Kawaguchi, H., Sakota, Y., Otonari, J. and Nitahara, H. (1993). Effects of polyphenols, including flavonoids, on glutathione S-transferase and glutathione reductase. *Biosci. Biotech. Biochem.* **57**, 1678-1680.

Jepson, P.C. and Sherratt, T.N. (1996). The dimensions of space and time in the assessment of ecotoxicological risks. In: Baird, D.J., Maltby, L., Greig-Smith, P.W. and Douben, P.E.T. (Eds.) *ECOtoxicology: Ecological Dimensions*. Chapman and Hall, London, pp.43-54.

Kapeja, E., Janusz, W., Zając, K.P., and Żurek, R. 1990. Stan zanieczyszczenia powietrza atmosferycznego w rejonie Kombinatu Górniczo-Hutniczego "Bolesław". *Zeszyty naukowe Akademii Górniczo-Hutniczej w Krakowie*, **32**: 11-20.

Karoly, E.D., Rose, R.L., Thompson, D.M., Hodgson, E., Rock, G.C., and Roe, R.M. (1996). Monooxygenase, esterase, and glutathione transferase activity associated with azinphosmethyl resistance in the tufted Apple bud Moth, *Playnota idaeusalis*. *Pestic. Biochem. Physiol.* **55**, 109-121.

Kedziorski, A., Nakonieczny, M., Migula, P., and Grzeskiak, M. (1996). Age-dependent activity patterns of carboxylesterases in insects exposed to cadmium. *Studia Societatis Scientiorum Torunensis* **4**, 71-76.

Khalil, M.A., Donker, M.H., Van Straalen, N.M. (1995). Long-term and short-

term changes in the energy budget of *Porcellio scaber Latreille* (Crustacea) exposed to cadmium polluted food. *Eur. J. Soil Biol.* **31**, 163-172.

Kimball, K.D. and Levins, S.A. (1985). Limitations of laboratory bioassays: the need for ecosystem-level testing. *BioScience* **35**: 165-71.

Kirby, M.L., Young, R.J. and Ottea, J.A. (1994). Mixed-function oxidase and glutathione S-transferase activities from field-collected larval and adult tobacco budworms, *Heliothis virescens* (F.). *Pestic. Biochem. Physiol.* **49**, 24-36.

Knigge, T. and Köhler H.-R. (2000). Lead impact on nutrition, energy reserves, respiration and stress protein (hsp 70) level *in Porcellio scaber* (Isopoda) populations differently preconditioned in their habitats. *Environ. Pollut.* **108**, 209-217.

Konno, Y. and Shishido, T. (1992). Distribution of glutathione S-transferase activity in insect tissues. *Appl. Entomol. Zool.* **27**, 391-397.

Kostaropoulos, I., Mantzari, A.E., and Papadopoulus, A.I. (1996). Alterations of some glutathione S-transferase characteristics during the development of *Tenebrio molitor* (Insecta: Coleoptera). *Insect Biochem. Molec. Biol.* **26**, 963-969.

Kramarz, P. (1999). The dynamics of accumulation and decontamination of cadmium and zinc in carnivorous invertebrates. 1. *Poecilus cupreus* L. *Bull. Environ. Contam. Toxicol.* **63**, 531-537.

Kramarz, P. and Laskowski, R. (1999). Effect of zinc contamination on life history parameters of a ground beetle, *Poecilus cupreus. Bull. Environ. Contam. Toxicol.* **59**, 525.

Laskowski, R., Pyza, E., Maryanski, M., Niklinska, M. (1998) Test on centipede *Lithobius mutabilis* L. Koch 1852 (Chilopoda: Lithobiidae). In: H. Løkke, C.A.M. van Gestel (eds.) Handbook of soil invertebrate toxicity tests. John Wiley & Sons Ltd, Chichester, 181-196.

Lawrence, A. and Poulter, C. (1996). The potential role of the estuarine amphipod, Gammarus duebeni, in sub-lethal ecotoxicology testing. *Water Science Technol.* **34**, 93-100.

Lindqvist, L. and Block, M. (1998). Sex specific interactions between Cd and Zn in three beetle species (Insecta, Coleoptera). *Bull. Environ. Contam. Toxicol.* **61**, 51-56.

Mantel, N. (1966). Evaluation of survival data and two new rank order statistics arising in its consideration. *Cancer Chemotherapy Reports* **50**, 163-170.

Matsubara, F., Nakayama, Y. and Masui, H. (1982). Influence of heavy metals on aseptically reared silkworm larvae (VI). Amount of accumulated Cd in the tissues and organs by the administration of Cd, Cd+Zn, and Cd+EDTA. *Environ. Control Biol.* **20**, 35.

Merrington, G., Winder, L. and Green, I. (1997). The uptake of cadmium and zinc by the bird-cherry oat aphid *Rhopalosiphum padi* (Homoptera: Aphididae) feeding on wheat grown on sewage sludge amended agricultural soil. *Environ. Pollut.* **96**, 111-114.

Migula, P. and Głowacka, E. (1996). Heavy metals as stressing factors in the red wood ants (*Formica polyctena*) from industrially polluted forests. *Fresenius J. Anal. Chem.* **354**, 653-659.

Migula, P., Kafel, A. Kedziorski, A. and Nakonieczny, M. (1989). Combined and separate effects of cadmium, lead and zinc on growth and feeding in the house cricket (Acheta domesticus). *Biologia* **44**, 911.

Mineau, P., Biomarkers: are there linkages to ecological effects? (1998), in *Multiple Stresses in Ecosystems*. Cech, J.J., Wilson, B.W. and Crosby, D.G. Eds., Lewis Publishers, Boca Raton, 92.

Mitton, J.B. (1997). Enzyme kinetics: quantitative analyses of enzyme performance. In *Selection in Natural Populations*. Oxford University Press, 21-26.

Newman, M.C. and McCloskey, J.T. (1996). Time-to-event analyses of ecotoxicity data. *Ecotoxicology* **5**, 187-196.

Oppenoorth, F.J. (1985). Biochemistry and genetics of insecticide resistance. Ed. G.A. Kerkut and L.J. Gilbert. *Comprehensive Insect Physiology, biochemistry and pharmacology*. Vol. 12, Pergamon, New York, pgs. 731-773.

Ortel, J. Effects of lead and cadmium on chemical composition and total water content of the pupal parasitoid, *Pimpla turionellae*. *Entomol. Exp. Appl.* **52**, 83.

Posthuma, L. and Van Straalen, N.M. (1993). Heavy-metal adaptation in terrestrial invertebrates: a review of occurrence, genetics, physiology and ecological consequences. *Comp. Biochem. Physiol.* **106C**, 11-38.

Read, H.J., Wheater, C.P., and Martin, M.H. (1987). Aspects of the ecology of Carabidae (Coleoptera) from woodlands polluted by heavy metals. *Environ. Pollut.* **48**, 61-76.

Rowe, C.L., Kinney, O.M., Nagle, R.D. and Congdon, J.D. (1998). Elevated maintenance costs in an anuran (Rana catesbeiana) exposed to a mixture of trace elements during the embryonic and early larval periods. *Physiol. Zool.* **71**, 27-35.

Rowe, C.L., Hopkins, W.A., Zehnder, C. and Congdon, J.D. (2001). Metabolic costs incurred by crayfish (*Procambarus acutus*) in a trace element-polluted habitat: further evidence of similar responses among diverse taxonomic groups. *Comp.e Biochem. Physiol.* **129C**, 275-283.

Santos, M.H., Troca da Cunha, N. and Bianchini, A. (2000). Effects of copper and zinc on growth, feeding and oxygen consumption of *Farfantepenaeus*

paulensis postlarvae (Decapoda: Penaeidae). *J. Exp. Marine Biol. Ecol.* **247**, 233-242.

Scharf, M.E., Meinke, L.J., Wright, R.J., Chandler, L.D. and Siegfried, B.D. (1999). Metabolism of carbaryl by insecticide-resistant and –susceptible western corn rootworm populations (Coleoptera: Chrysomelidae). *Pestic. Biochem. Phyisol.* **63**, 85-96.

Sherratt, T.N. and Jepson, P.C. (1993). A metapopulation approach to modelling the long-term impact of pesticides on invertebrates. *J. Appl. Ecol.* **30**, 696.

Sibly, R.M. and Calow, P. (1989). A life-cycle theory of responses to stress. *Biol. J. Linn. Soc.* **37**, 101-116.

Spicer, J.I. and Weber, R.E. (1991). Respiratory impairment in crustaceans and molluscs due to exposure to heavy metals. *Comp. Biochem. Physiol.* **100C**, 339-342.

Sprague, J.B. and Fogels, A. (1977). Watch the Y in Bioassay, in *Proceedings, 3rd Aquatic Toxicity Workshop*, Environmental Protection Technical Report No. EPS-5-AR-77-1, Halifax, Canada, 107-115.

Stefanowicz, A. (2001). Przestrzenne zroznicowanie mikrosidlisk glebowych w terenach skazonych I nieskazonych metalami ciezkimi. BSc Thesis, Faculty of Chemistry, Jagiellonian University, Krakow, Poland.

Stone, D., Jepson, P., Kramarz, P. and Laskowski, R. (2001a). Time to death response in carabid beetles exposed to multiple stressors along a gradient of heavy metal pollution. *Environ. Pollut.* **113**, 239-244.

Stone, D., Jepson, P. and Laskowski, R. (2001b). Detoxification enzymes as indicators of physiological stress in heavy metal exposed ground beetles (Coleoptera: Carabidae). *Comp. Biochem. Physiol. C* (submitted).

Strojan, C.L. (1978). The impact of zinc smelter emissions on forest litter arthropods. *Oikos* **31**, 41.

Suter, G.W., Sample, B.E., Jones, D.S, and Ashwood, T.L. (1994). *Approach and strategy for performing ecological risk assessments for the U.S. Department of Energy's Oak Ridge Reservation*. Report ES/ER/TM-33/R1, Oak Ridge National Laboratory, Oak Ridge, TN.

Suter, G.W. (1995). Endpoints of interest at different levels of biological organization. In *Ecological Toxicity Testing: scale, complexity and relevance*. Cairns, J. and Niederlehner, B.R., Eds. Lewis Publishers, Boca Raton, FL, 38-39.

Tabashnik, B.E. and Johnson, M.W. (1999). Evolution of pesticide resistance in natural enemies. In: Bellows, T.S. and Fisher, T.W. (Eds.) *Handbook of Biological Control*. Academic Press, London, pp. 673-689.

Tranvik, L. Bengtsson, G. and Rundgren, S. (1993). Relative abundance and resistance traits of two Collembola species under metal stress. *J. Appl. Ecol.* **30**, 43-52.

Walker, C.H., Hopkin, S.P., Sibly, R.M. and Peakall, D.B. (2001). Evolution of resistance to pollution. In: *Principles of Ecotoxicology*. Taylor and Francis, London, pp. 220-221.

Wicklum, D. and Davies, R.W. (1996). The effects of chronic cadmium stress on energy acquisition and allocation in a freshwater benthic invertebrate predator. *Aquatic Toxicol.* **35**, 237-252.

Widdows, J. and Donkin P. (1991). Role of physiological energetics in ecotoxicology. *Comp. Biochem. Physiol.* **100C**, 69-75.

Wilczek, G. and Migula, P. (1996). Metal body burdens and detoxifying enzymes in spiders from industrially polluted areas. *Fresenius J. Anal. Chem.* **354**, 643-647.

Wilczek, G., Majkus, Z., Migula, P., Bednarska, K., and Swierczek, E. (1997). Heavy metals and detoxifying enzymes in spiders from coal and metallurgic dumps near Ostrava (Czech Republic). *Proc. 16th Europ. Coll. Arachnol.*, 317-328.

Yu, S.J. (1996). Insect glutathione S-transferases. *Zool. Stud.* **35**: 9-19.

Zachariassen, K.E., Aunaas, T., Børseth, J.F., and Einarson, S. (1991). Physiological parameters in ecotoxicology. *Comp. Biochem. Physiol.* **100C**, 77-79.

Appendix

Measures of Population Increase after Exposure to Chronic and Acute Xenobiotics in Pea Aphids (Homoptera: Aphididae)

Rysard Laskowski, David Stone and Paul Jepson

Submitted to Environmental Entomology

Entomological Society of America

March 2001

Abstract:

Pea aphids (*Acyrthosiphon pisum* Harris) were raised on potted bean plants grown on soil treated with cadmium chloride (0, 100, and 200 mg kg^{-1}), imidacloprid (0.4 and 40 g a.i. ha^{-1}) or a mixture of chemicals in a factorial design. Three separate experiments were performed for suitability in ecotoxicological assays: (1) a full Life Table Response Experiment (LTRE); (2) a short-term bioassay conducted on adult aphids; and (3) a short-term bioassay conducted on neonate aphids. Following each experiment, population increase rates (λ) were calculated. In single chemical treatments, significant negative effects on λ were observed for imidacloprid (NTN) and 200 mg kg^{-1} Cd in the LTRE. In both short-term bioassays, only the highest dose of NTN caused a significant decrease in λ. Unexpectedly, there was a positive effect of cadmium (200 mg kg^{-1}) and NTN (40 g a.i. ha^{-1}) mixture on λ compared to NTN alone or in combination with 100 mg kg^{-1} Cd. In contrast to both short-term bioassays, the LTRE at the highest dose of NTN and NTN combined with 100 mg kg^{-1} Cd caused λ to decrease to below 1.0, indicating possible extinction. Adult short-term bioassays were compared with juvenile short-term bioassays for suitability in ecotoxicological assessment.

Introduction:

Selecting appropriate endpoints to determine the impact of a xenobiotic on an organism is essential for ecotoxicological assays. Currently, the most frequent bioassay calculates a lethal dose for fifty percent of individuals (LC_{50}) exposed to a single chemical. This approach is unable to detect effects at population levels or consider reproductive impairment. To address these deficiencies, it has been suggested that full life table response experiments (LTRE) be used to calculate the intrinsic rate of population increase as a more sensitive indicator of potential adverse effects.

The LTRE incorporates parameters of developmental rate, fecundity and longevity, assuming conditions exist in an unlimited environment. Endpoints such as the survivorship of a cohort of organisms, and the per capita birth rates of females are included in the LTRE calculation. The LTRE has been conducted on several species to assess the effects of acute (Day and Kaushik, 1987) and chronic xenobiotics (Wong and Wong, 1990; Allan and Daniels, 1982). Bechmann (1994) compared the LTRE with the traditional LC_{50} for the effects of copper on the copepod *Tisbe furcata*. The LTRE detected negative demographic effects occurring far below the LC_{50}, further supporting the enhanced sensitivity of population measures compared with traditional approaches. Despite the advantages of utilizing the LTRE, many ecotoxicologists have not employed this method due to constraints in time, expense and labor associated with obtaining all the required measurements.

In response to these disadvantages in the LTRE, the use of short-term bioassays to calculate the instantaneous rates of growth (r_i) were investigated as an alternative method. The instantaneous rate of growth measures the exponential increase of a population over a selected time period. The r_i has been used for assessing natural populations (Kareiva and Sahakian, 1990) but has not found widespread use in ecotoxicology. Walthall and Stark (1997a) compared LTRE and r_i methods using pea aphids, *Acyrthosiphon pisum* Harris, exposed to the insecticide imidacloprid. They observed that after 11 days, the LTRE and r_i were not significantly different from one another. This suggests that given sufficient time, r_i could be used as an alternate endpoint in ecotoxicological studies. However, this is dependent upon the life history of the test organism and the mode of action, persistence and duration of exposure to the xenobiotic(s).

The purpose of this study was to test the suitability of r_i vs. LTRE in assessing the ecotoxicological impact of cadmium, imidacloprid and the mixture of both xenobiotics on the pea aphid (*A. pisum*). *A. pisum* was chosen because of its short generation time, high reproductive rate, significance as a pest species, and the detailed comparative study by Walthall and Stark (1997a).

The toxicants used include an acute xenobiotic (imidacloprid) designed to kill rapidly and degrade versus a chronic pollutant (Cd) that accumulates slowly and does not degrade. Imidacloprid (NTN) belongs to the chloronicotinyl class of insecticides. Unlike conventional insecticides that act

on muscarinic receptors or the Na^{2+} channels, NTN acts as an agonist to the insect nicotinic acetylcholine receptors. This novel mode of action presents an attractive option to combat insect resistance and augment integrative pest management programs. NTN is especially active in sucking and mining pests, including aphids, and has excellent systemic properties as a seed treatment. It is speculated that NTN is the forerunner to a class of compounds that will find wider use in agricultural applications (Leicht, 1996).

Contamination of ecosystems by Cd can occur by many sources including the application of sludge and fertilizers to agricultural fields, atmospheric deposition, or industrial activity. In addition, crops growing in contaminated areas may translocate Cd into stem and foliar regions. Crawford et al. (1995) has demonstrated that aphids feeding on contaminated plants accumulate Cd. Given the increased concentrations of Cd in the environment and the growing use of NTN in agroecosystems, an investigation into the population effects caused by each xenobiotic and as a mixture, is relevant today.

Materials and Methods:

Test organisms and chemicals:

Pea aphids (*Acyrthosiphon pisum*) were obtained from a laboratory stock culture kept at the Oregon State University. The aphids were bred on potted broad bean plants, *Vicia faba* L., in a controlled temperature room at 18

°C with a photoperiod of 16 hr: 8 hr light:dark regimen. Imidacloprid (NTN) was formulated product (emulsifable concentrate) containing 17.4% active ingredient (Bayer Corporation, Kansas City, MO). Imidacloprid was applied at a rate equivalent to 4 and 40 g a.i. ha^{-1}. Cd was applied to the soil in soluble form as cadmium chloride.

Chemical treatment:

The broad bean plants were grown in 9.5×9.5 cm plastic pots. The pots were filled with 250 g (dry weight) of a garden soil. Before potting the plants, the soil was oven-dried at 105°C and the water-holding capacity was measured gravimetrically. The soil was watered with 100 ml cadmium chloride solution or distilled water and four beans were planted per plot. Concentrations of cadmium chloride were adjusted to reach the final nominal Cd concentrations in soil of 100 or 200 mg kg^{-1} dry weight soil. After three to four weeks, when the plants were 15-30 cm high, imidacloprid (NTN) was added in 50 ml distilled water per pot to obtain 4 and 40 g a.i. ha^{-1}, approximately equivalent to 0.01 and 0.1 recommended field dose, respectively. All non-insecticide treatments received 50 ml of distilled water. Adult or juvenile aphids were introduced to the plants 4 days after the application of NTN. Throughout the experiment the pots were kept in plastic trays filled with tap water. Watering the soil surface was avoided to minimize potential leaching of cadmium chloride from the soil.

Experimental method:

In order to compare results from the full life table response experiment (LTRE) and two different short-term bioassays, three separate experiments on Cd and imidacloprid toxicity were performed. In the LTRE (EXP1), adults of reproductive age were placed individually into clip cages (one per plant). After 24 hours, the adults and all but one neonate were removed from the cages. Each clip cage was monitored daily until the test aphid died. All neonates were counted and removed. To minimize the effects of plant quality, each clip cage was moved to a new leaf when the old one started to wither. Each pot, with 2 or 3 clip cages, was treated as one replicate and 4 to 8 (control) replicates were used per treatment. The intrinsic finite rates of increase (λ_{intr}) values were calculated for each pot (Equation 1).

The second experiment (EXP2) was designed to estimate rapid effects on aphid populations using a short-term bioassay. The plants were prepared as described above and four days after NTN treatment, 5 or 10 adult aphids (depending on the plant size) of reproductive age were placed in each pot. The plants in each test pot were enclosed in a transparent plastic sleeve. The sleeve contained openings covered with netting to provide ventilation and to minimize fungal growth. After 7 days, the plants were cut and the aphids were immobilized by cold temperature and counted. The instantaneous rate of increase was calculated for each pot (Equation 2).

The third experiment (EXP3) was similar to EXP2 but utilized neonates instead of adults. To begin EXP3, several adult aphids were isolated onto enclosed plants. The following day, neonates were collected from the plants and used for the duration of the experiment. Five or ten neonates were placed on plants in each pot and left for 12 days. The length of the experiment was chosen following the results obtained by Walthall and Stark (1997) mentioned earlier. Both EXP2 and EXP3 were run using 8 replicates per treatment.

Calculations and statistical methods:

In LTRE, the intrinsic rates of population increase, r_{intr}, were calculated from the Euler-Lotka formula using the age-specific daily survival rates, l_x, and fecundities, m_x:

$$1 = \sum_{1}^{36} l_x m_x e^{-rx} \quad . \qquad \text{(Equation 1)}$$

The units of x are age in days (from day one to 36, the maximum lifespan of aphids tested), and e is the base of natural logarithm. The r_{intr} values were recalculated to $\lambda_{intr}=e^r$. Using λ rather than r has the advantage of obtaining results that are interpretable for populations that resulted in death without leaving any progeny ($\lambda=0$).

Data from EXP 2 and EXP 3 were used to calculate instantaneous rates of population increase, expressed as λ_{inst}:

$$\lambda_{inst} = \left(\frac{N_t}{N_0}\right)^{\frac{1}{t}}, \qquad \text{(Equation 2)}$$

where N_t and N_0 are the population sizes at the start and at the end of experiment respectively and t is time in days.

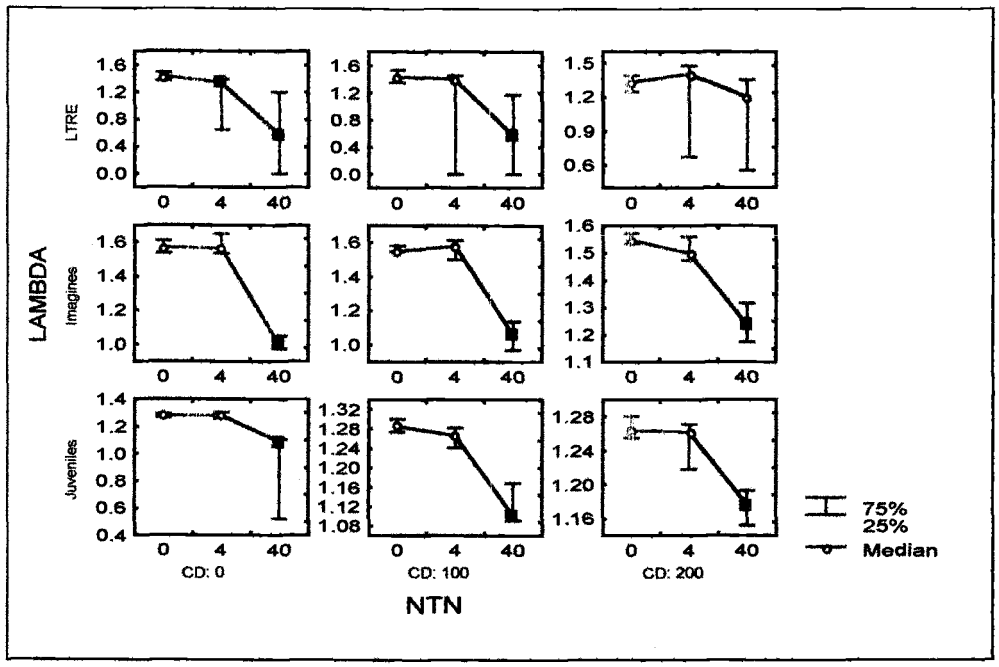
The distributions of calculated λ values were checked for normality with the Kolmogorov-Smirnov test. Due to the fact that the distributions in EXP1 were significantly different from normal and no transformation could normalize them, the treatment effects were compared using the Mann-Whitney non-parametric test. Comparisons were made for imidacloprid effects across three different Cd concentrations and for Cd effects across three doses of imidacloprid. For a particular concentration of Cd, two imidacloprid treatments (4 and 40 g a.i. ha^{-1}) were compared against the treatment without NTN. Similarly, for a particular dose of NTN, the two Cd concentrations (100 and 200 mg kg^{-1}) were compared against the treatment with no Cd added. Data were plotted as medians with 25 – 75th percentile intervals.

Results:

All experiments revealed significant effects of imidacloprid on the fitness of aphids. The LTRE revealed that both doses of NTN (4 and 40 g a.i. ha^{-1}) in the absence of Cd caused a significant decrease in λ_{intr}. The short-term bioassays resulted in a noticeable effect only at the highest dose of insecticide. When mixed with Cd at 100 mg kg^{-1}, a significant decrease in λ was found only at 40 g a.i. ha^{-1} NTN in all experiments. At the highest dose of Cd (200 mg kg^{-1}), significant effects of imidacloprid relative to Cd-200/NTN-0 were found only in the short-term bioassays, although at this treatment all λ_{intr} values were lower than in control populations (Fig. A.1).

The effect of Cd was less pronounced compared with imidacloprid and a significant decrease in fitness was found only for 200 mg kg^{-1} Cd (without insecticide) in the LTRE. However, at the highest dose of imidacloprid, cadmium at 200 mg kg^{-1} caused a substantial positive effect in all three experiments. This effect was demonstrated by an increase in aphid performance in comparison to NTN treatment without Cd. The effect was significant in both short-term bioassays (Fig. A.2).

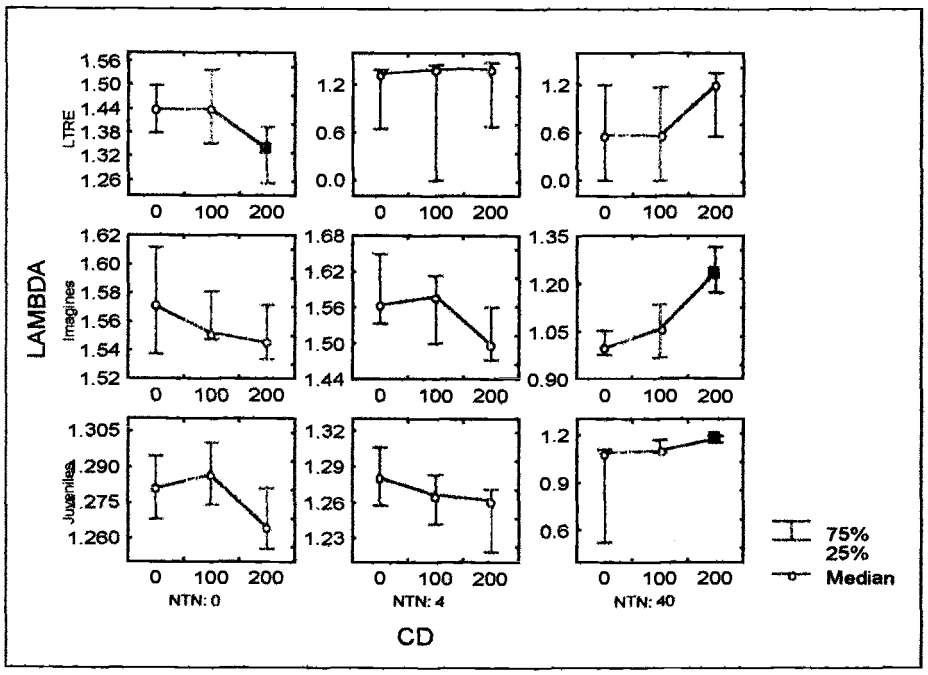
Figure A.1. Effect of imidacloprid (NTN) treatment at three different levels of cadmium (Cd) pollution on intrinsic rate of population increase (λ_{intr}; LTRE) and instantaneous rate of increase (λ_{inst}) in experiments initiated with adult aphids and neonates. Treatments significantly different from NTN-0 (no imidacloprid) at the respective cadmium level are marked with filled black squares. Note differences in scale between plots.



In the LTRE, the highest dose of imidacloprid caused λ_{intr} to decrease to 0.58 without Cd and 0.57 when mixed with Cd (Table A.1 and A.2). A $\lambda <$ 1 indicates potential population extinction at these treatments if the effects were persistent.

In contrast, none of the median λ_{inst} values dropped below 1.0 (although at the 0/40 treatment the average was 0.831). Thus, interpretation of

Figure A.2. Effect of cadmium (Cd) at three different levels of imidacloprid (NTN) treatment on intrinsic rate of population increase (λ_{intr}; LTRE) and instantaneous rate of increase (λ_{inst}) in experiments initiated with adult aphids and neonates. Treatments significantly different from Cd-0 (no cadmium addition) at the respective imidacloprid level are marked with filled black squares. Note differences in scale between plots.



the short-term bioassay results could lead to the opposite conclusion - none of the treatments used would cause population extinction.

Discussion:

This study emphasized the greater degree of information obtainable from population-level endpoints versus traditional bioassays. In the LTRE assay, detailed information was attained by calculating realized fecundity, life

Table A.1. Effect of cadmium and imidacloprid on aphid fitness, λ_{intr}, in the LTRE; N = number of replicate cultures (pots).

treatment		N	λ_{intr}	
Cd[mgkg^{-1}]	NTN[g a.i.ha^{-1}]		Average±S.D.	Median
0	0	8	1.436±0.07	1.44
0	4	4	1.023±0.68	1.345
0	40	4	0.603±0.70	0.58
100	0	5	1.438±0.10	1.44
100	4	5	0.872±0.80	1.4
100	40	4	0.585±0.68	0.565
200	0	5	1.268±0.21	1.34
200	4	4	1.078±0.72	1.41
200	40	4	0.953±0.65	1.2

span and onset to reproduction in pea aphids. In addition to analyzing more parameters, population-level endpoints can detect unexpected effects as well. For example, Walthall and Stark (1997b) found that the LC_{60} was not a good predictor of population level effects based on comparison of a 72-hr LC_{60} versus population level endpoints for aphids exposed to imidacloprid. The LTRE detected a "reservoir effect" in which surviving aphids were able to maintain high reproductive rates and compensate for the loss of susceptible individuals. Thus, the traditional approach may overestimate the efficacy of insecticides in pest control.

Table A.2. Effect of cadmium and imidacloprid on aphid fitness, λ_{inst}, in the 7-day bioassay with adult aphids (EXP2) and the 12-day bioassay with new-born nymphs (EXP3); number of replicate cultures (pots) = 8 for all treatments.

Treatment		EXP2 [λ_{inst}]		EXP3 [λ_{inst}]	
Cd[mgkg^{-1}]	NTN[g a.i.ha^{-1}]	Average±S.D.	median	Average ±S.D.	median
0	0	1.578±0.04	1.57	1.280±0.02	1.28
0	4	1.588±0.10	1.565	1.284±0.03	1.28
0	40	1.025±0.10	1.0	0.831±0.51	1.09
100	0	1.561±0.02	1.55	1.284±0.03	1.29
100	4	1.558±0.07	1.58	1.265±0.03	1.27
100	40	1.035±0.13	1.06	1.108±0.09	1.105
200	0	1.550±0.03	1.545	1.255±0.04	1.265
200	4	1.514±0.06	1.5	1.249±0.04	1.26
200	40	1.243±0.09	1.235	1.173±0.03	1.18

To further exploit the benefits of population-level endpoints in assessing biological responses to pollutants, we investigated the influence of chronic xenobiotics, acute xenobiotics and mixtures. In our experiments, both the LTRE and the short-term bioassays were able to detect a significant difference in λ at the highest dose of imidacloprid compared with controls. The LTRE was able to detect a decrease in λ_{intr} at the lower dose of insecticide, whereas there was no significant difference detected by the short-term bioassays at this dose. This suggests that the LTRE may be more sensitive as

an indicator of population-level effects exposed to lower doses of acute xenobiotics.

In assessing the effects of Cd alone, neither the LTRE nor the short-term bioassays were able to detect an effect at the lowest dose (100 mg kg^{-1}). In addition, both adult and juvenile short-term bioassays were unable to detect significant effects at 200 mg kg^{-1} Cd. In contrast, the LTRE demonstrated a significant difference in λ at the high dose of Cd compared with controls. Based on these results, the LTRE may be more suitable when testing population-level effects for chronic xenobiotics that accumulate slowly in the organism. Negative effects may not be visible in short-term bioassays simply due to the fact that a critical concentration is not reached or the test ends too quickly. Slow accumulation of metals was demonstrated in several studies (Janssen 1991, Witzel 1998, Kramarz 1999), and in some species the equilibrium level did not appear to have been reached (Janssen 1991, Neuhauser *et* al. 1995, Kramarz 1999, Spurgeon and Hopkin 1999).

In determining the effect of Cd on λ for aphids exposed to varying doses of imidacloprid, juvenile and adult short-term bioassays indicated significant differences at the highest dose of insecticide and heavy metal. The LTRE did not detect significant effects, although there was a decrease in λ_{intr}. Both LTRE and short-term bioassays demonstrated significant effects of NTN on aphid fitness exposed to 100 mg kg^{-1} Cd and 40 g a.i. NTN ha^{-1} compared with controls. However, only the short-term bioassay was able to detect a

significant effect of NTN at the highest dose of Cd (200 mg kg^{-1}). In this case, the short-term bioassays were more sensitive in detecting population effects based on mixtures of Cd and imidacloprid.

Additional advantages of the short-term bioassay compared with the LTRE include the reduction in time, labour and expense involved in experimentation. To further optimize the short-term bioassay, a comparison was made between using adult and juvenile aphids. The adult aphid bioassay required less time (7 days vs. 12 days) and was less labour intensive due to the ease of handling large adults versus juveniles. An advantage of the juvenile assay is that the exact age and origin of the aphids is assured. Analysis of the results indicated that the adult and juvenile short-term bioassays were similar in determining significant differences in λ for mixtures and individual xenobiotics. This suggests that the adult short-term bioassay may be a suitable alternate to using juveniles to assess population level effects.

The LTRE predicted extinction at two treatments with λs of 0.58 and 0.57 (40 g a.i. NTN alone and in combination with 100 mg kg^{-1} Cd, respectively). Neither short-term bioassay resulted in a λ below 1.0 for any treatment. This may indicate that ultimately, the LTRE has a greater sensitivity than the short-term bioassays. However, the LTRE was associated with a greater degree of variation in calculating λ compared with both short-term bioassays. This variability resulted from using only two or three aphids per replicate. Due to the large amount of time required to handle single aphids

in clip cages, increasing numbers of aphids would be difficult in full factorial experiments. Also, the high sensitivity of LTRE does not necessarily mean high ecological realism. Based on their studies on the toxicity of Margosan-O on pea aphids, Stark and Wennergren (1995) noted that the population-level effects of this insecticide would not be predicted correctly by evaluating only one life stage even with LTRE approach. Instead, they suggested the use of a stage-structured approach as a more adequate demographic alternative to ecotoxicological studies.

When extrapolating these results to other studies, it should be considered that aphids reproduce parthenogenetically and were exposed by feeding on systemic xenobiotics through the plant phloem. A test organism with important differences in life history and exposure route may yield conflicting results. Summarizing the results of this study and those obtained by other authors we suggest that:

(1) Short-term bioassays can be used for studies on population effects of pesticides and other fast-acting and biodegradable toxicants. However, they are not suitable for testing non-degradable chemicals of moderate toxicity and with a tendency to accumulate in an organism's body during its lifetime.

(2) Among short-term bioassays, those made on neonates offer similar results to those made on adults. The advantage of the latter is easier handling and shorter test. However, the most adequate results should be obtained with a population of stable age distribution if possible.

(3) The long-term studies, preferably full LTREs, offered more sensitive results in tests on toxicity of non-degradable chemicals that tend to accumulate both in the environment and in test organisms. Toxic effects of such chemicals may not be detectable in short-term bioassays.

(4) In ecotoxicological studies, it is crucial to investigate experiments on interactions between chemicals that may exist in the same environment. In particular, studies on interactions between pesticides and metals have been seriously neglected, possibly due to the simple fact that very few ecotoxicologists have sufficient expertise or interest in these different groups of chemicals.

References:

Allan, J.D. and Daniels, R.E. (1982). Life table evaluation of chronic exposure of *Eurytemora affins* (Copepoda) to kepone. *Mar. Biol.* **66**, 179-184.

Bechmann, R.K. (1994). Use of life tables and LC50 tests to evaluate chronic and acute toxicity effects of copper on the marine copepod *Tisbe furcata* (Baird). *Environ. Toxicol. Chem.* **13**, 1509-1517.

Crawford, L.A., I.D. Hodkinson and Lepp, N.W. (1995). The effects of elevated host-plant cadmium and copper on the performance of the aphid Aphis fabae. *J. Appl. Ecol.* **32**, 528-535.

Day, K. and Kaushik, N.K. (1987). An assessment of the chronic toxicity of the synthetic pyrethroid, fenvalerate, to *Daphnia galeata mendoate*, using life tables. *Environ. Pollut.* **44**, 13-26.

Janssen, M.P.M. (1991). Comparison of cadmium kinetics in four soil arthropod species. *PhD Thesis, Vrije University, Amsterdam*, pp. 75-89.

Kareiva, P. and Sahakian, R. (1990). Tritrophic effects of a simple architectural mutation in pea plants. *Nature* **345**, 433.

Kramarz, P. (1999). The dynamics of accumulation and decontamination of cadmium and zinc in carnivorous invertebrates. 2. The centipede Lithobius mutabilis Koch. *Bull. Environ. Contam. Toxicol.*, **63**, 531-537.

Leitch, W. (1996). Imidacloprid – a chloronicotinyl insecticide; biological activity and agricultural significance. *Pflanzenschutz-Nachrichten Bayer* **49**, 71-84.

Neuhauser, E.F., Cukic, Z.V., Malecki, R.M. Loehr, R.C. and Durkin, P.R. (1995). Bioconcentration and biokinetics of heavy metals in the earthworm. *Environ. Pollut.* **3**, 293-301.

Spurgeon, D.J. and Hopkin, S.P. (1999). Comparison of metal accumulation and excretion kinetics in earthworms (*Eisenia fetida*) exposed to contaminated field and laboratory soils. *Appl. Soil. Ecol.* **11**, 227-243.

Stark, J.D. and Wennergreen, U. (1995). Can population effects of pesticides be predicted from demographic toxicological studies? *J. Econ. Entomol.* **88**, 1089-1096.

Walthall, W.K. and J.D. Stark (1997a). Comparison of two population-level ecotoxicological endpoints: the intrinsic and instantaneous rates of increase. *Environ. Toxicol. Chem.* **16**, 1068-1073.

Walthall, W.K. and J.D. Stark (1997b). A comparison of acute mortality and population growth rate as endpoints of toxicological effect. *Ecotoxicol. Environ. Saf.* **37**, 45-52.

Witzel, B. (1998). Uptake, storage and loss of cadmium and lead in the woodlouse *Porcellio scaber* (Crustacea, Isopoda). *Water Air Soil Pollut.* **108**, 51-68.

Wong, C.K. and P.K. Wong. 1990. Life table evaluation of the effects of cadmium exposure on the freshwater cladoceran, *Moina macrocopa. Bull. Environ. Contam. Toxicol.* **44**,135-141.