Elemental Distribution in Reproductive and Neural Organs of the *Epilachna nylanderi* (Coleoptera: Coccinellidae), a Phytophage of Nickel Hyperaccumulator *Berkheya coddii* (Asterales: Asteraceae) by micro-PIXE

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**ABSTRACT.** The phenomenon of metal hyperaccumulation by plants is often explained by a pathogen or herbivore defense hypothesis. However, some insects feeding on metal hyperaccumulating plants are adapted to the high level of metals in plant tissues. Former studies on species that feed on the leaves of *Berkheya coddii* Roessler 1958 (Asteraceae), a nickel-hyperaccumulating plant, demonstrated several protective mechanisms involved in internal distribution, immobilization, and elimination of Ni from the midgut and Malpighian tubules. These species are mainly coleopterans, including the lady beetle, *Epilachna nylanderi* (Mulsant 1850) (Coleoptera: Coccinellidae), collected from the ultramafic ecosystem near Barberton in South Africa. By performing particle-induced X-ray emission microanalysis elemental microanalysis (PIXE), this study examined whether Ni may be harmful to internal body systems that decide on insect reactivity (central nervous system [CNS]), their reproduction, and the relationships between Ni and other micronutrients. Data on elemental distribution of nine selected elements in target organs of *E. nylanderi* were compared with the existing data for other insect species adapted to the excess of metals. Micro-PIXE maps of seven regions of the CNS showed Ni mainly in the neural connectives, while cerebral ganglia were better protected. Concentrations of other bivalent metals were lower than those of Ni. Testis, compared with other reproductive organs, showed low amounts of Ni. Zn was effectively regulated at physiological dietary levels. In insects exposed to excess dietary Zn, it was also accumulated in the reproductive organs. Comparison of *E. nylanderi* with other insects that ingest hyperaccumulating plants, especially chrysomelid *Chrysolina clathrata* (Clark) (Coleoptera: Chrysomelidae), showed lower protection of the CNS and reproductive organs.

**Key Words:** Ni-hyperaccumulating plant, elemental distribution, X-ray microanalysis, nuclear microprobe, plant-insect interaction

The phenomenon of metal hyperaccumulation by plants is often explained by the elemental defense hypothesis (Boyd 2004, 2007; Boyd and Martens 1998). This hypothesis suggests that maintaining high levels of heavy metal in aboveground organs of the plant may have evolved to defend hyperaccumulating plants against some natural enemies, such as herbivores and pathogens. To counteract this plant defense, some herb-grazing insects have evolved various physiological, ecological, and behavioral strategies to minimize toxic effects of metals in their diet (Vijver et al. 2004). Because of these adaptations, some herbivorous insects can use hyperaccumulating plants as the major part of their diet (Mesjasz-Przybyłowicz and Przybyłowicz 2001, Peterson et al. 2003, Boyd 2004, Jhee et al. 2006, Migula et al. 2007, Boyd 2009). Knowledge of the physiological mechanisms driving metal tolerance in insects is still limited. Studying specific mechanisms of metal tolerance will broaden understanding of coevolution between hyperaccumulating plants and their grazers in ultramafic ecosystems, and ecosystems contaminated with metals. An excess of consumed metals might become toxic, and these insects must prevent, decrease, or repair the effects of metals that have entered their bodies.

*Berkheya coddii* Roessler 1958 (Asterales: Asteraceae), an endemic plant species from ultramafic ecosystems, is one of five Ni hyperaccumulators from South Africa, for which several associated herbivorous insect species able to utilize metalliferous plant material without any significant effects on their population dynamics have been reported (Morrey et al. 1989, Mesjasz-Przybyłowicz and Przybyłowicz 2001, Migula et al. 2007, Boyd 2009). Among them, two coleopteran species—the chrysomelid leaf beetle (*Chrysolina clathrata* (Clark), (Coleoptera: Chrysomelidae), formerly *Chrysolina pardalina* Fabricius, 1781) and the lady beetle (*Epilachna nylanderi*) (Mulsant 1850) (Coleoptera: Coccinellidae)—and the grasshopper (*Stenosepa* sp.) have been the subjects and the objects of intensive structural, physiological, and ecological studies, including elemental microanalysis using the particle-induced X-ray emission microanalysis (PIXE) (Mesjasz-Przybyłowicz et al. 2002; Przybyłowicz et al. 2003, 2004, 2005; Augustyniak et al. 2002, 2006, 2008; Nakonieczny 2007; Migula et al. 2011). Elemental distributions in the whole body and in the isolated body organs were obtained (Przybyłowicz et al. 2004). In previous studies, the importance of the target organs as the first line of defense against excessive amounts of metals has been demonstrated. Several structural and morphological adaptations to utilize the Ni-rich leaves of *B. coddii* were found in *C. clathrata* and *Stenosepa* sp. The most important structural and functional adaptations took place in the midgut and Malpighian tubules. Most of the consumed nickel is directly rejected from the gut. Ni accumulated in granular concretions of the epithelial midgut cells is eliminated during apoptosis of these cells (Klag et al. 2002; Przybyłowicz et al. 2004, 2005; Augustyniak et al. 2006, 2008; Nakonieczny 2007). *E. nylanderi* belongs to the group that easily modifies their food preferences (Giorgi et al. 2009). Former studies on this species searched for mechanisms involved in distribution, immobilization, and decontamination of nickel from the midgut and Malpighian tubules and other forms of Ni elimination (Przybyłowicz 2006, 2008; Nakonieczny 2007).
Toxicokinetic processes help to determine how much of a toxic substance reaches insect target organs, where the substances entered the organism, and the internal distribution and metabolism of these substances (Migula 1996, Przybyłowicz et al. 2004, Vijver et al. 2004, Mesjasz-Przybyłowicz and Przybyłowicz 2011). Excess of Ni may change the balance between microelements in the internal organs of insects (Mesjasz-Przybyłowicz et al. 2002, Migula et al. 2004, Augustyniak et al. 2008). Both species of beetles mentioned above can maintain Ni body levels that are toxic to related species that feed on nonaccumulating Ni plant species (Nakonieczny 2007). Detailed analysis using the micro-PXIE method indicated an uneven distribution of metals in C. clathrata, from 0.8 μg g⁻¹ of Ni in the fat body to 3,000 μg g⁻¹ in the Malpighian tubules (Przybyłowicz et al. 2003, 2005; Nakonieczny 2007). Analysis of Ni concentration and distribution in the reproductive organs and brain ganglia of this species indicated that these organs were sufficiently protected against Ni toxicity (Przybyłowicz et al. 2003). It is not known whether the distribution of trace elements in the open circulatory system of E. nylanderi may be harmful to other body organs responsible for life history traits and reproductive potential.

The aim of this study is to examine whether both the internal organs that control fast functional responses (central nervous system [CNS]) and the reproduction of insects are effectively protected against excess dietary nickel, and to determine the relationships between Ni and other micronutrients. Quantitative and qualitative data on elemental distribution in these target organs of E. nylanderi were compared with the existing data for other insect species associated with Ni-hyperaccumulating plant species.

Materials and Methods

Adult beetles of E. nylanderi were collected from the ultramafic outcrops near Barberton (Mpumalanga Province, South Africa) as described elsewhere (Augustyniak et al. 2002, Migula et al. 2011). E. nylanderi underwent four instars and complete development within 17–31 d. Adults emerge after 3 wk of pupation and continue feeding on B. coddii leaves, consuming only their soft epidermal and parenchymal parts (Migula et al. 2011).

Sample Preparation for Elemental Microanalysis. Material was sectioned from insects starved for 24 h to empty their guts of the remains of ingested food. Similar to former studies describing elemental distribution in the gut and Malpighian tubules, the CNS and the male internal reproductive organs were isolated from the same group of beetles for further analysis (Przybyłowicz et al. 2005, Nakonieczny 2007, Migula et al. 2011).

Zn and Ni, according to the biological classification of Nieboer and Richardson (1980), belong to the border class of metals that have no binding preference and form ligands with many functional groups (sulfur containing or oxygen containing). Ni interacts with Zn, which in high concentrations is also toxic. Replacement of Zn by Ni may lead to enhanced toxicity. On the other hand, Zn in excess may exert a protective role against the stimulatory role of Ni in production of hydroxyl radicals (Nakonieczny 2007). This was the reason why a group of insects was also kept in the laboratory on B. coddii leaves presoaked in tap water containing 0.1 mM Zn for 1 wk, to study how an additional stressor might affect the redistribution of elements in the analyzed samples.

Isolated internal organs were immediately preserved by cryofixation against a cold metal block and freeze-dried. Lyophilized samples were mounted between the two formvar film layers (Przybyłowicz et al. 2005).

Elemental Microanalysis. Microanalyses were carried out using the nuclear microprobe at the Materials Research Department, iThemba LABS, South Africa, as described previously (Prozovsky et al. 1995; Przybyłowicz et al. 1999, 2005). A proton beam of 3-MeV energy, generated by the 6-MV single-ended Van de Graaff accelerator, was focused to a 3 by 3 μm² spot and raster scanned over selected areas of analyzed samples, using square or rectangular scan patterns with a variable number of pixels (up to 128 by 128). Proton current was restricted to 200–300 pA to minimize specimen beam damage. Particle-induced X-ray emission (PIXE) and proton backscattering spectrometry (BS) were used simultaneously. PIXE spectra were registered with an Si(Li) Link PentaFet detector (80-mm² active area and 8-μm Be window) with an additional 190-μm Kapton layer as an external absorber. The effective energy resolution of the PIXE system (for the Mn Kα line) was 165–170 eV, measured for individual spectra. The detector was positioned at a take-off angle of 135° and a working distance of 25 mm. The X-ray energy range was set between 1 and 36 keV. BS spectra were recorded with an annular Si surface barrier detector (100-μm thick) positioned at an average angle of 176°. Data were acquired in the event-by-event mode. The normalization of results was done using the integrated beam charge and collected simultaneously from a Faraday cup located behind the specimen and from the insulated specimen holder. The total accumulated charge per scan varied from 1 to 5 μC. Overall, 53 scans were performed.

Quantitative results were obtained by a standardless method using GeoPIXE II software package (Ryan et al. 1990, Ryan 2000). The error estimates are extracted from the error matrix generated in the fit, and the minimum detection limits are calculated using the Currie equation (Currie 1968). The detailed calibration of detector efficiency, the thicknesses of selectable X-ray attenuating filters, and studies on the accuracy and precision have been reported elsewhere (Van Achterbergh et al. 1998). The procedure reported there was used for the Link Si(Li) detector used in this study. The calibration of the analytical system was tested by measurements of standards—pure elements and synthetic glasses with known quantities of selected minor elements (internal standards), the X-ray peaks of which cover practically the whole measurable energy range. Quantitative elemental mapping was performed using a dynamic analysis method (Ryan and Jamieson 1993, Ryan et al. 1995, Ryan 2000). This method generates elemental images that are 1) overlap resolved; 2) with subtracted background; and 3) quantitative, i.e., accumulated in mg kg⁻¹ dry weight units. Maps were constructed using matrix composition and areal density representative of the scanned area.

In addition, average elemental concentrations from specific selected regions drawn over the main structures of dissected body parts of adult E. nylanderi were obtained. These concentrations were obtained using a full nonlinear deconvolution procedure to fit PIXE spectra (Ryan et al. 1990), with matrix corrections based on areal density and matrix composition obtained from the corresponding BS spectra, fitted with a RUMP simulation package (Doolittle 1986) with non-Rutherford cross-sections for C, O, and N. Matrix corrections done on the basis of BS results were essential due to the variable thickness of analyzed specimens. Elemental concentrations from these areas are also reported in mg kg⁻¹ dry weight.

Of 16 measured elements, the data for 9 elements (S, Cl, K, Ca, Fe, Ni, Mn, Cu, and Zn), for which all levels were above the accepted 99% detection limits, were presented in maps and appropriate tables, and interpreted.

Statistical procedures were performed using the Statistica v.10 software package for personal computer. The means and SD were calculated from multiple scans across the 3 by 3 grid. Significance of means within the same element and between the species was analyzed using one-way analysis of variance and least significant difference tests, at P < 0.05.

Results

The Central Nervous System. The CNS of E. nylanderi is similar to other beetle species and consists of the brain with three fused ganglionic masses (proto-, deutro-, and tritocerebrum), three ventral ganglia (pro-, meso-, and metathoracic), abdominal ganglia, and the ventral
connectives; N4, paired giant connectives; N5, second thoracic gland; N6, third thoracic gland; N7, part of the hindgut. Different letters denote significant differences between selected regions at 

Selected regions are as shown in Figure 1: N0, left optic lobes and protocerebrum; N1, deuto- and tritocerebrum; N2, first thoracic gland; N3, paired giant ganglia; N4, paired giant connectives; N5, second thoracic gland; N6, third thoracic gland; N7, part of the hindgut. Micro-PIXE visualization of the distribution of nine elements in selected regions of the CNS is shown in two rows. Scale = 1 mm (white bars on the left side in the images of the upper row) and 0.1 mm (white bars on the right side of the images in the bottom row).

The distribution pattern of Cl was similar to that of S. Concentration of Cl was higher in the areas of interganglionic neural connectives (N3 and N4), but its concentration was much lower in the areas of interganglionic connectives (N3 and N4) and the first thoracic gland (N2). These structures were characterized by the highest Ca levels. Mn concentrations in a given area was not higher than 50%. Concentrations of Mn in the CNS was 251.2 \( \mu \text{g} \text{g}^{-1} \) and the highest levels were in the neural connectives and the third thoracic gland. The mean coefficient of variation of Mn was maintained in the main brain ganglia and in the thoracic ganglia (Table 1). The lowest values were found in the areas of interganglionic connectives (N3, N4) and the first thoracic gland (N2). These structures were characterized by the highest Ca levels. Mn concentrations were the lowest of all examined elements. The coefficient of variation for Mn (0.34) was three times higher than for Ca (0.11). The concentrations of Mn in the examined neural structures was kept at similar, insignificant levels. The average concentration of Ni in the examined neural structures was kept at similar, insignificant levels. The average concentration of Ni in the examined neural structures was kept at similar, insignificant levels. The average concentration of Ni in the examined neural structures was kept at similar, insignificant levels. The average concentration of Ni in the examined neural structures was kept at similar, insignificant levels.

Table 1. Concentration of selected elements (mean ± SD in mg kg\(^{-1}\)) in various parts of the central nervous system of the adult *Epilachna nylaneri*

| Region | S     | Cl   | K     | Ca   | Mn   | Fe   | Ni   | Cu   | Zn   |
|-------|-------|------|-------|------|------|------|------|------|------|
| N0    | 4,620 ± 830a | 1,550 ± 14a | 11,080 ± 92a | 292 ± 21a | 1.3 ± 0.8a | 96 ± 2a | 144 ± 3a | 247 ± 3a | 57 ± 1a |
| N1    | 4,720 ± 840a | 2,050 ± 140b | 13,970 ± 120c | 251 ± 18a | 4 ± 2a | 114 ± 4a | 161 ± 6a | 506 ± 12b | 59 ± 4a |
| N2    | 3,990 ± 810a | 1,270 ± 116a | 9,320 ± 100b | 316 ± 28a | 10 ± 2b | 110 ± 3a | 248 ± 8b | 211 ± 6a | 60 ± 6a |
| N3    | 8,700 ± 1,400a | 2,150 ± 270b | 10,300 ± 220a | 340 ± 66a | 12 ± 5b | 114 ± 12a | 420 ± 38c | 255 ± 35a | 25 ± 15 |
| N4    | 11,200 ± 1,700b | 2,080 ± 350b | 9,010 ± 140b | 488 ± 73b | 14 ± 7b | 84 ± 10a | 518 ± 27c | 156 ± 18a | 40 ± 14a |
| N5    | 4,510 ± 790a | 795 ± 99c | 12,840 ± 63c | 162 ± 22c | 3.6 ± 0.8a | 87 ± 2a | 147 ± 2a | 17 ± 1c | 49 ± 2a |
| N6    | 5,030 ± 930a | 840 ± 110c | 14,910 ± 97c | 129 ± 24c | 2 ± 1a | 91 ± 2a | 122 ± 3a | 11.6 ± 1c | 53 ± 2a |
| N7    | 4,440 ± 1,070a | 930 ± 160c | 10,300 ± 110a | 287 ± 21a | 4 ± 1a | 101 ± 2a | 395 ± 6c | 12 ± 1c | 53 ± 2a |

Data are obtained from PIKE spectra from these regions using a full nonlinear deconvolution procedure. Detection limits (99%) are given in parentheses.

The first image on the left: light micrograph of lyophilized sample with selected regions: N0, left optic lobes and protocerebrum; N1, deuto- and tritocerebrum; N2, first thoracic gland; N3, paired giant ganglia; N4, paired giant connectives; N5, second thoracic gland; N6, third thoracic gland; N7, part of the hindgut. Different letters denote significant differences between selected regions at \( P < 0.05 \).
Table 2. Concentration of selected elements (mean ± SD in mg kg⁻¹) in testes of the adult Epilachna nylanderi

| Region | S        | Cl      | K       | Ca      | Mn      | Fe      | Ni      | Cu      | Zn      |
|--------|----------|---------|---------|---------|---------|---------|---------|---------|---------|
| T0     | 10,400 ± 630a (130) | 5,060 ± 180 (21) | 15,000 ± 480a (4.6) | 237 ± 49a (3.1) | 3.4 ± 0.4a (0.8) | 46 ± 1 (0.6) | 3.3 ± 0.5 (0.8) | 143 ± 2a (0.8) | 85 ± 3a (0.8) |
| T1     | 16,500 ± 1,000b (310) | 5,810 ± 220 (46) | 11,800 ± 410b (8.6) | 440 ± 46b (5.9) | 1.2b | 57 ± 2 (1) | 3.2 ± 0.7 (1.3) | 360 ± 3b (1.4) | 131 ± 3b (1.5) |

Selected regions cover distal (T0) and basal (T1) regions of follicles. Data are obtained from PIXE spectra from these regions (shown in Fig. 2) using a full nonlinear deconvolution procedure. Detection limits (99%) are given in parentheses. Different letters denote significant differences between selected regions at P < 0.05.

Discussion

Effects of dietary Ni in insects associated with Ni-hyperaccumulating plants are difficult to compare with those in Ni-sensitive insects. Tests of choice showed that such plant material is unpalatable to most species. Their starvation induces degeneration processes; body weight is lost and it leads to insect death. Nakonieczny (2007) compared structural and functional adaptations of C. clathrata to an Ni-rich diet (leaves of B. coddii) with Chrysotina herbacea kept on Ni-enriched leaves of Mentha species. C. herbacea restricted feeding at 1,000 times lower concentration of Ni in the diet than C. clathrata and was not able to eliminate excessive Ni from the body. Its detoxifying system was also less effective than that of C. clathrata. Zawisza-Raszk and Doleżył (2008) showed that fewer than 50% of the Spodoptera exigua larvae fed Ni-contaminated leaves (900 mg kg⁻¹) reached the pupal stage. Similar Ni effects were observed in the leaf-mining larvae, Eriocrania semipurpurella (Kozlov et al. 2000). The low rate of Ni bioaccumulation of this species was concomitant with increased concentrations of the metal, decreased efficiency of feeding, and reduction of the body weight.

Nickel and Essential Micronutrients in E. nylanderi. The trace elements’ feeding requirements are known only for a few insect species (Nation 2001). Insects have evolved various physiological mechanisms that regulate levels of essential ions such as K, Ca, Mn, Zn, Fe, or Cu (Bagatto and Shorthouse 1996). All elements (except Ni) analyzed in E. nylanderi were essential micronutrients. Body levels of these metals did not deviate much from those of other insects (Mattson and Scriber 1986).

Organisms must maintain adequate supplies of Fe to meet nutritional requirements while controlling their potential toxic property. Iron is transported to hemolymph by the transferrin and stored in the non-toxic ferritin. This complex is distributed further to various compartments of an insect cell (Nichol et al. 2002). When in excess, iron produces reactive oxygen species that may damage cellular membrane components or nucleic acids (Nichol et al. 2002, Green et al. 2010).
Concentrations of Fe in organs of E. nylanderi do not differ from those in another coleopteran species—C. clathrata (Table 4). Beanland et al. (2003) found that another ladybird species, Epilachna varivestis, gained weight proportionally to the presence of Fe and Zn. Fe concentrations in other groups of Ni-adapted insects were organ dependent. In the brain of grasshoppers, Stenoseca sp., it was only 64.8 ± 0.3 mg kg⁻¹ (Augustyniak et al. 2008). Nearly the same Fe concentrations were found in the brain of Chorthippus brunneus (Orthoptera) inhabiting uncontaminated grassland (Augustyniak et al. 2006). Similar Fe concentrations were found in the head sap feeders of B. coddii: an aphid, Protaphis pseudocardui (62 ± 0.2 mg kg⁻¹), and a leafhopper, Norialsus berkheyae (43 ± 2 mg kg⁻¹) (Migula et al. 2007). The observations imply that regulatory mechanisms of Fe in Ni-adapted insects are not species specific.

Green et al. (2010) stated that if dietary Zn is given in excess to lady beetles, metal concentrations remain in the body at rather constant levels, whereas Ni and Cu are quickly rejected. This finding was not confirmed in the case of E. nylanderi exposed to high Zn dietary levels, for which excess of this metal was an additional stressor accumulating at high levels in the target organs (Table 4). The elimination rate of zinc from the insect body is high (Kramarz 1999). In case of E. nylanderi, the time left for depuration of the gut (24 h) was too short for the effective bioelimination of the metal.

Copper was present at levels high enough for the proper functioning of the reproductive organs and the CNS. These concentrations were much higher than reported in other coleopteran species inhabiting metal-contaminated areas (Migula et al. 2004). Comparisons with C. clathrata demonstrated similarities of the Cu burden in the reproductive organs and in the brain ganglia but differences in Malpighian tubules, confirming regulatory importance of this organ in maintaining proper levels of the metal (Table 4). It is worth mentioning that at high concentrations, copper can also participate in the production of free radicals (Valko et al. 2005, Das et al. 2008).

Nickel in Target Organs of E. nylanderi—A Comparison With Other Insect Species. Ni is generally a nonessential element in insect physiology. In insect species living in Ni-uncontaminated areas, it is weakly regulated. This metal could reach cells as a cotransporter in calcium channels. Accumulation of Ni depends on the genes responsible for specific metal transporter protein 1 synthesis (Nunez et al. 2010). Low Fe or Cu levels upregulate expression of this gene (Chung et al. 2004, Troade et al. 2010).

Insect species living on ultramafic sites have had enough time to cope with an excess of Ni and evolve various physiological, morphological, and behavioral adaptations allowing control of Ni levels. The main strategy used by E. nylanderi against excess dietary Ni is its effective direct elimination from the gut. Ni is inactivated in granular concretions present in the midgut epithelial cells. These short-lived cells could easily move through apoptotic or necrotic pathways and are eventually eliminated with feces. Lost cells are quickly replaced by the new ones developed from regenerative cells, rich in number, in the midgut epithelium (Rost-Roszkowska et al. 2008, 2010; Migula et al. 2011). They could also use a similar mechanism, as described in C. clathrata, which effectively eliminates Ni through transmembrane transport: from the anterior part of midgut into the hindgut with the help of the Malpighian tubules (Przybyłowicz et al. 2005, Nakonieczny 2007).

Elemental maps of E. nylanderi testis (Fig. 2) indicated that this organ is well protected against excess Ni and at the same time maintains concentrations of other elements allowing homeostasis, necessary either of the part of intensive division of the germ cells (T0) or in the areas of their differentiation and transportation to seminal vesicles (T1). Despite this, quantitative relations between Ni and other determined metals differ between analyzed organs. Compared with testis (Fig. 4; Table 2), the mean concentrations of Ni in other selected reproductive organs were more than 11 and 17 times higher in insects kept on a diet without and with an excess of Zn, respectively (Table 4). Worth mentioning is that successful protection of generative organs against excess of dietary Ni in

**Fig. 3.** The first image on the top: light micrograph image of the reproductive system of the adult Epilachna nylanderi male with selected regions: R0, a whole region covering male reproductive organs (without testes)—accessory gland with vas deferens, seminal vesicles, vas efferens, and ejaculatory duct; R1, vas deferens, vas efferens, and ejaculatory duct; R2, seminal vesicles; R3, accessory gland. The images below: Micro-PIXE visualization of the distribution of nine elements in selected regions of the male reproductive system. Scale (white bar on the top right side of each image) = 0.1 mm.
Detection limits (99%) are given in parentheses. Different letters denote significant differences between selected regions in Figure 3, covering male reproductive organs (without testes); R1, vas deferens, vas efferens, and ejaculatory duct; R2, seminal vesicles; R3, accessory gland.

Comparison with the data obtained for E. nylanderi than in the midgut and at similar levels as in the Malpighian tubules (Table 3).

Other male reproductive organs also had lower Ni levels in the accessory reproductive structures, suggesting better protection against Ni than that of E. nylanderi (Table 4).

Ni concentrations in the brain ganglia of E. nylanderi were lower than in the midgut and at similar levels as in the Malpighian tubules (Tables 3 and 4). Comparison with the data obtained for C. clathrata (Migula et al. 2011) suggests worse protection of the brain against Ni toxicity (Table 4). Total elimination of the remains of quickly coagulating hemolymph from external structures of the examined CNS was not possible without washing the isolated organs. The higher concentration of Ni found in the region of interganglionic neural connectives probably also came from a coagulated hemolymph (N3 and N4; Figs. 1 and 5).

**Table 3. Concentration of selected elements (mean ± SD in mg kg⁻¹) in various parts of reproductive system of the adult male of Epilachna nylanderi**

| Region | S        | Cl       | K        | Ca       | Mn       | Fe       | Ni       | Cu       | Zn       |
|--------|----------|----------|----------|----------|----------|----------|----------|----------|----------|
| R0     | 3,890 ± 510 | 926 ± 44  | 4,310 ± 26b | 2,610 ± 9c | 157 ± 2c  | 135 ± 1a  | 39 ± 1c  | 13.6 ± 0.7b | 2.760 ± 29c |
| R1     | 3,260 ± 600 | 861 ± 50  | 5,840 ± 53a | 8,400 ± 57a | 520 ± 5a  | 179 ± 4b  | 111 ± 4a | 23 ± 2a  | 10,940 ± 120a |
| R2     | 3,660 ± 390 | 850 ± 300 | 2,700 ± 17c | 414 ± 9d  | 49 ± 0.6d | 90 ± 0.8c | 12 ± 0.4d | 7.5 ± 0.4c | 75 ± 0.9d |
| R3     | 3,350 ± 560 | 821 ± 55  | 5,050 ± 33a | 5,630 ± 20b | 335 ± 4b  | 137 ± 3a  | 74 ± 3b  | 19 ± 2a  | 7,050 ± 77b |

Data are obtained from PIXE spectra from these regions using a full nonlinear deconvolution procedure. Selected regions: R0, whole region of the map shown in Figure 3, covering male reproductive organs (without testes); R1, vas deferens, vas efferens, and ejaculatory duct; R2, seminal vesicles; R3, accessory gland. Detection limits (99%) are given in parentheses. Different letters denote significant differences between selected regions at P < 0.05.

**Table 4. Average mineral element concentrations (mean ± SD in mg kg⁻¹) in selected parts of the leaf of Berkheya coddii (B.c.) and various internal organs of Epilachna nylanderi (E.n.) and Chrysolina clathrata (C.c.)**

| Species | Region         | S         | Cl     | K       | Ca       | Mn       | Fe       | Ni       | Cu       | Zn       |
|---------|----------------|-----------|--------|---------|----------|----------|----------|----------|----------|----------|
| B.c.    | Total leaf     | ND        | 35,950 | 5,730   | 7,850    | 99       | 609      | 23,110   | 82       | 32       |
| B.c.    | Mesophylla     | 4,550 ± 160 | 40,000 | 52,300  | 76,000   | 20 ± 10  | 113 ± 3.5 | 17,3 ± 46 | ND       | 50 ± 30  |
| E.n.    | Midgut         | 2,900 ± 661* | 3,700 ± 157* | 1,120 ± 157* | 3,000 ± 66* | 100 ± 1.2* | 11 ± 3.6* | 324 ± 9* | 10 ± 2.2* | 2,100 ± 104* |
| C.c.    | Malpighian tubes | 6,580 ± 504 | 5,180 ± 123 | 14,060 ± 331 | 235 ± 18 | 4 ± 2      | 129 ± 6  | 16 ± 2   | 36 ± 3   | 64 ± 5   |
| C.c.    | Malpighian tubes | 1,460 ± 567* | 14,750 ± 110* | 8,770 ± 65* | 7,790 ± 56* | 386 ± 8  | 114 ± 4.2* | 126 ± 4.2* | 11 ± 2* | 2,130 ± 31* |
| E.n.    | Brain ganglia   | 4,670 ± 123* | 1,800 ± 246* | 12,520 ± 113* | 271 ± 47 | 2.6 ± 3.9* | 105 ± 6  | 152 ± 15* | 376 ± 10.5 | 583 ± 7.5 |
| C.c.    | Testes         | 9,270 ± 369 | 6,790 ± 164 | 16,100 ± 324 | 386 ± 10 | 1.5 ± 0.5 | 121 ± 3  | 6.9 ± 0.5 | 7.3 ± 0.5 | 39 ± 2 |
| E.n.    | Testes         | 8,200 ± 1,410 | 4,080 ± 260 | 13,650 ± 103* | 341 ± 28* | 3 ± 0.6   | 57 ± 1.5 | 4 ± 0.7  | 296 ± 4.5 | 122 ± 2.5* |
| C.c.    | Other male     | 6,250 ± 140 | 6,310 ± 111 | 16,430 ± 433 | 107 ± 16 | 2.7 ± 0.5 | 77 ± 2   | 5 ± 1    | 230 ± 5  | 77 ± 1   |
| E.n.    | Other male     | 2,540 ± 749 | 4,550 ± 210* | 14,770 ± 131* | 1,320 ± 6* | 8.6 ± 1.3* | 110 ± 2.7 | 66 ± 2* | 9.7 ± 1.3* | 82 ± 2.7 |
| E.n.b.  | Reproductive organs | 3,420 ± 515 | 844 ± 46* | 4,530 ± 32* | 4,810 ± 24* | 302 ± 6.7* | 135 ± 2.2 | 56 ± 2.1* | 17 ± 3.4 | 6,020 ± 57* |
| C.c.    | Reproductive organs | 6,820 ± 266 | 3,520 ± 78 | 13,100 ± 255 | 245 ± 13 | 1.6 ± 0.9 | 81 ± 2   | 12 ± 1   | 29 ± 2* | 136 ± 4 |

ND, not determined.

*Significant differences between the species (P < 0.05).

Data from Mesjasz-Przybyłowicz et al. (2001) and Mesjasz-Przybyłowicz et al. (2004).

Insects fed on leaves enriched with Zn.

Fig. 4. Mean concentration of Ni (mg kg⁻¹) in selected regions of Epilachna nylanderi male reproductive tract. Explanation of the abbreviated names of the regions are given in Figures 2 and 3. Different letters denote significant differences between the organs at P < 0.05.
These structures, as well as the ganglia of the CNS, are well protected from direct inflow of external metals through an effective brain–blood barrier. The border between blood and neurons is about 10 times thicker than those separating nerve cells of mammals (Nation 2001). This is why the concentrations of ions on the external side of neuronal membrane and inside the neurons could differ from those in the hemolymph. The open insect circulatory system forces a strong isolation of the neuronal microenvironment using a network of fluid-filled clefts connected to the glial cells. Any disturbance of mineral transport could also influence the neurosecretory function of the main ganglia and may affect a variety of the physiological functions of the insect. This is also the reason why high concentrations of K determined in this study reflect external, not internal, levels in the analyzed samples.

Low bioconcentration ratio (bioconcentration factor) of Ni in the analyzed organs of E. nylanderi suggests that insects can efficiently deal with excessive amounts of metal. The low Ni concentration (86 mg kg$^{-1}$) was also reported in the brain of grasshopper (Stenoscopa sp.) larvae. Micro-PIXE analysis indicated less Ni in the optic lobe of the brain (31 mg kg$^{-1}$) than in the cerebral ganglia (246 mg kg$^{-1}$; Augustyniak et al. 2008). These results agree with the pattern of Ni distribution found in the CNS of E. nylanderi (Fig. 2). It is worth adding that only traces of Ni (1.4 mg kg$^{-1}$) were found in the heads of the thrips, Haplothrips acanthoscelis, the species that feeds on the leaves of B. coddii by puncturing them and sucking up liquid concentrations (Przybyłowicz et al. 2004).

Micro-PIXE analyses made possible comparisons of the quantitative variability and interactions of metals in different organs of E. nylanderi. The Ca/Ni ratio was very high in testis (87.4) and other internal male reproductive organs (73.1), whereas in the CNS, it was nearly balanced (1.1). This seems logical because more Ca in the reproductive system is needed to support intensive metabolic processes generated by cell division and the synthesis of various compounds supporting vitality of the germ cells and their transport within the reproductive tracts (Nation 2001). A similar effect was observed for Cu for which the concentration in the testis is more than 70 times higher than that of Ni. The highest Fe/Ni ratio in the testis (14.6) could also be related to the transport mechanisms of metals and Fe metabolism (Nichol et al. 2002). Insects kept on a diet without surplus of Zn had a low Zn/Ni ratio, suggesting that Zn was replaced by Ni, with possible adverse effects on enzymes that require Zn as a cofactor ( Migula et al. 2004, Augustyniak et al. 2006).

The time of total development of E. nylanderi in similar laboratory conditions is nearly twice as long as observed for C. clathrata, 50.6 versus 25.0 d (Nakonieczny 2007). In comparison with C. clathrata, micro-PIXE analysis indicated weaker tolerance of the first species to the excess of Ni in the diet, reflected by higher concentrations of this metal in reproductive and neural organs. These differences may derive from various phylogenetic developments of the compared insects. C. clathrata represents chrysomelid beetles, which developed earlier as herbivores, whereas among generally carnivorous coccinellids, E. nylanderi represents a monophyletic group of herbivorous Epilachninae.

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Fig. 5. Mean concentration of Ni (mg kg$^{-1}$) in selected regions of E. nylanderi CNS. Explanation of the abbreviated names of the regions is given in Figure 1. Different letters denote significant differences (P < 0.05) between selected parts of the CNS.
