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USE OF SELECTED ASPECTS OF *Cepaea nemoralis* (Gastropoda: Pulmonata) POLYMORPHISM IN ENVIRONMENTAL BIOMONITORING ON LUBLIN AREA

Danuta Kowalczyk-Pecka, Robert Stryjecki

Department of Zoology, Ecology and Wildlife Management, University of Life Sciences in Lublin
Akademicka str. 13, 20-950 Lublin, danakp@wp

Summary. An analysis was made of the amount of accumulated iron, lead, and zinc in various tissues of *Cepaea nemoralis* snails and in plant and soil samples from six habitats in Lublin and its vicinity, characterized by varying degrees of human impact. A total of 28 colour forms were identified, indicating a high degree of polymorphic variation in the species at these sites. Analysis of content of the metals in body parts of snails of different colour types suggests that these metals may influence regulation of expression of phenotypic traits, and confirms that this *Cepaea nemoralis* is useful in the search for biomarkers of exposure.

Key words: *Cepaea nemoralis*, polymorphism, heavy metals, bioaccumulation, environmental monitoring, bioindicator

INTRODUCTION

There have been numerous approaches to explaining the basis of the polymorphism of *Cepaea nemoralis*. This problem has been the subject of observations and research for many years. The genetic conditioning of this variation is relatively well known [Lamotte 1988, Cameron 1992, Cook and Pettit 1998 Ożgo 2005a, b], whereas the genetic basis of the pigmentation of slugs is known in only a few species [Reise 1997, Jordaens 2001, 2006a, b]. Yet to be resolved are questions concerning the factors which induce the genes responsible for colour variation in snails and slugs. These may be environmental, climatic, or habitat factors, including chemical compounds and elements in soil and food.

The polymorphism of the grove snail *Cepea nemoralis* is a very convenient subject for ecological research due to the visible differences in the background colour and number of bands on the shell. This species often occurs in spatially isolated populations. In Poland, *Cepaea nemoralis* was originally present only in Western Pomerania, but has now been introduced or accidentally transferred to habitats throughout nearly the entire country, where it usually lives as a synanthrope [Wąsowski and Penkowski 2003, Wiktor 2004]. As first-order consumers in the food chain, snails are an excellent source of information concerning the transfer of environmental pollutants from the soil through plants to animal organisms, which has been described in numerous publications [Gomot and Pihan 1997, Swaileh *et al*. 2000, Yasoshima *et al*. 2001, Beeby and Richmond 2002, Nahmanii and Rossi 2003, Notten *et al*. 2005, 2006, 2008].

The aim of the study was to compare the content of iron, lead, and zinc in the soft tissues and shells of similarly-pigmented *Cepaea nemoralis* snails from six habitats characterized by different degrees of human impact, in order to confirm the suitability of this species as a bioindicator organism. An attempt was made to analyse the potential effect of metal accumulation on the expression of traits of colour polymorphism in snails from one habitat.

MATERIAL AND METHODS

The adult *Cepaea nemoralis* (Linnaeus, 1758) snails used in the study were collected by hand in the first week of June, 2012, from six habitats characterized by varying degrees of human impact, located in Lublin and its vicinity. The sites were ranked according to their pollution levels, determined *a priori* based on their distance from roads and traffic intensity in the area, and *a posteriori* based on metal content in the leaves of *Urtica dioica* and in the soil. The sites were designated as follows: S1 – 51°16'7"N, 22°34'24"E, Ponikwoda-Bazylianówka district; S2 – 51°14'59"N, 22°34'21"E – southern part of Stare Miasto-Podzamcze; S3 – 51°13'57"N, 22°29'15"E – Węglin; S4 – 51°14'2"N, 22°33'37"E – behind the sugar refinery; S5 – 51°13'36"N, 22°37'46"E – Felin; S6 – 51°10'10"N, 22°30'40"E – Zemborzyce (district names are given according to the website of the City Council of Lublin).

In accordance with methodological guidelines for research on grove snail polymorphism, open habitats without bushes or trees were selected, with vegetation as homogeneous as possible, so that the collection sites would be comparable. The collection area did not exceed 400 m^2. Shells were classified into seven colour variants: pale yellow – PY, yellow – Y, pale pink – PP, pink – P, dark pink – DP, pale brown – PB, and brown – B. Also taken into account was the absence of dark bands on the shell, designated as 00000, or the number of bands, i.e. 1 designated as 00300, 3 – 00345, 5 – 12345, and 5 merged bands – (12345) [Ożgo 2005b].

In addition to snails, samples of plants (*Urtica dioica*) and soil were collected from the habitats investigated. The plant leaves were stored at -25°C until analysis. The soil was collected from an area of about 10 cm^2 after removal of the humus layer, and stored in closed containers in cold store.

Samples of snail tissue, soil, and plants were prepared according to a previously described procedure [Kowalczyk-Pecka 2009, Kowalczyk-Pecka and Czepiel-Mil 2011].

Iron, lead, and zinc content were analysed by atomic absorption spectrometry at the Central Analytical Laboratory of the University of Life Sciences in Lublin. For initial comparison of accumulated metals in the shells and soft tissues, the results were converted to µg per g DW of snail tissue and shells. Average values were compared using Tukey's test.

RESULTS AND DISCUSSION

Information on possible causes and consequences of the frequency and variability of colour forms in *Cepaea nemoralis* was collected and presented in a clear manner by Ożgo [2005a, b]. Apart from obvious genetic differences, important factors for the expression and frequency of colour variation may include natural visual selection by second-order consumers, apostatic selection favouring rare genotypes that are beneficial for the population, climate selection, acreage effects that mean dominance of particular colour forms over large areas irrespective of changing co-existing environmental factors, and heterosis [Ożgo 2005b]. At the molecular level, more reliable information on the polymorphism of these molluscs can be obtained only by analysis of DNA markers, not of allozymes. Snails exhibit very high variation of both allozymes and mitochondrial DNA [Backeliau *et al*. 1997, Ożgo 2005b].

Analysis of the material collected revealed high colour variation in the shells of *Cepea nemoralis* snails found in Lublin and its vicinity (Tab. 1). A total of 28 morphic forms were identified, of which 8 were found only at single sites. The most colour and band types, 19, were noted at S4, and the fewest, 5, at site S2. It is worth noting that in habitats S5 and S6, which were the least contaminated by the metals tested, 11 morphic types were found in each, although these differed between the two sites. The dominant background colour was light brown, occurring without bands and with one band at all of the sites investigated. The fewest snails had brown or dark pink shells.

In order to confirm the usefulness of *Cepea nemoralis* as bioindicator organisms *in vivo* in their natural habitats, snails with pale brown shells with one band were used, because this morphic form was present at all of the sites investigated.

The iron, lead, and zinc content determined in the dry weight of the shells and soft tissues of the snails shows a clear upward trend as the concentration of these metals in the plants and soil from the same habitats increased (Tab. 2). This is very likely associated with the increasing degree of human impact in the study sites. The amount of accumulated iron and zinc in the shells and soft tissues of the snails was markedly higher than the lead content in all samples. This reflects not only the amount of these pollutants in the environment, but also differences in the biotransformation processes of these metals and in detoxification mechanisms, which in snails are specific for different chemical compounds and elements. This may be why the amount of iron accumulated in the snail shells did not differ significantly from its content in the soft tissues, whereas the difference in the amount of lead, and particularly of zinc, in the shells and soft tissues was noticeable (Tab. 2). This may be because it takes much more time for zinc to be incorporated into the shell than into other tissues, or it may indicate that iron and lead are eliminated from the organism faster than zinc or other metals.

Table 1. Classification of colour forms of the grove snail *Cepaea nemoralis* in the habitats investigated

Background colour	Number of bands*	S1	S2	S3	S4	S5	S6
PY	00000	-	-	+	-	+	+
PY	00300	+	-	+	-	-	-
PY	00345	-	-	-	+	-	-
PY	12345	+	-	+	+	+	-
PY	(12345)	+	-	+	-	+	-
Y	00000	-	-	-	-	-	+
Y	00300	+	-	-	+	+	+
Y	00345	-	-	-	+	-	+
Y	12345	+	-	-	+	-	-
Y	(12345)	-	+	-	+	-	-
PP	00000	-	-	-	+	+	-
PP	00300	-	-	+	+	+	-
PP	00345	-	-	-	+	-	-
PP	12345	+	+	-	+	+	-
PP	(12345)	+	-	+	-	-	-
P	00000	-	-	-	+	-	+
P	00300	-	-	+	+	+	+
P	00345	-	-	-	+	-	+
P	12345	-	-	-	+	-	-
P	(12345)	-	+	-	+	-	-
DP	00300	-	-	-	-	-	+
DP	12345	-	-	-	-	-	+
PB	00000	+	+	+	+	+	+
PB	00300	+	+	+	+	+	+
PB	00345	-	-	-	-	-	+
PB	12345	-	-	-	+	+	-
PB	(12345)	-	-	-	+	+	-
B	00000	+	-	-	-	-	-

PY – pale yellow, Y – yellow, PP – pale pink, P – pink, DP – dark pink, PB – pale brown, B – brown;
*Designations for number of bands are explained in „Material and methods”
S1–S6 – site designations are given in „Material and methods”

Table 2. Content of metals in the bodies of *Cepaea nemoralis* snails (n = 10) with pale brown shells with one band – PB 00300, in plants (*Urtica dioica*), and in soil from the habitats investigated (µg/gDM)

Sample	S1	S2	S3	S4	S5	S6
SH-Fe	463.34 ± 10.12^{a}	388.21 ± 9.54^{b}	355.37 ± 8.77^{bc}	325.98 ± 5.98^{bc}	219.11 ± 4.03^{c}	212.76 ± 5.87^{c}
ST-Fe	411.54 ± 9.94^{a}	399.44 ± 10.02^{a}	371.09 ± 8.45^{ab}	336.78 ± 9.92^{b}	226.11 ± 9.65^{c}	198.67 ± 7.86^{cd}
UD-Fe	62.01 ± 3.78^{a}	52.56 ± 3.59^{b}	49.76 ± 3.56^{b}	44.21 ± 3.27^{bc}	42.16 ± 3.12^{bc}	40.12 ± 3.08^{bc}
S-Fe	4997.1 ± 87.23^{a}	4356.2 ± 80.11^{b}	4122.8 ± 74.43^{bc}	3828.2 ± 70.04^{c}	3767.7 ± 65.98^{c}	3625.3 ± 54.77^{c}
SH-Pb	0.186 ± 0.012^{a}	0.119 ± 0.007^{ab}	0.098 ± 0.008^{b}	0.087 ± 0.006^{b}	0.061 ± 0.004^{bc}	0.045 ± 0.002^{c}
ST-Pb	0.203 ± 0.012^{a}	0.184 ± 0.011^{ab}	0.168 ± 0.011^{b}	0.142 ± 0.012^{c}	0.083 ± 0.006^{d}	0.064 ± 0.004^{e}
UD-Pb	0.339 ± 0.031^{a}	0.325 ± 0.028^{a}	0.155 ± 0.022^{b}	0.128 ± 0.019^{b}	0.09 ± 0.005^{c}	0.045 ± 0.002^{d}
S-Pb	1.597 ± 0.06^{a}	1.465 ± 0.07^{a}	1.232 ± 0.07^{ab}	1.002 ± 0.05^{b}	0.712 ± 0.05^{c}	0.674 ± 0.04^{c}
SH-Zn	134.76 ± 5.98^{a}	126.06 ± 4.23^{a}	109.06 ± 3.55^{ab}	102.21 ± 3.41^{ab}	92.34 ± 3.87^{b}	87.05 ± 3.35^{b}
ST-Zn	862.98 ± 20.34^{a}	825.65 ± 18.87^{ab}	792.06 ± 18.12^{ab}	699.01 ± 16.81^{b}	624.87 ± 14.55^{bc}	597.12 ± 12.43^{c}
UD-Zn	25.76 ± 1.54^{a}	23.07 ± 1.43^{a}	21.87 ± 1.20^{b}	20.26 ± 1.12^{bc}	18.95 ± 1.12^{c}	17.43 ± 1.01^{c}
S-Zn	276.56 ± 6.44^{a}	212.54 ± 6.25^{ab}	124.98 ± 4.76^{b}	112.47 ± 4.27^{bc}	87.76 ± 3.98^{c}	67.98 ± 3.88^{d}

SH – shell, ST – soft tissues, UD – *Urtica dioica*, S – soil, S1–S6 Site designations are given in the „Materials and methods”
Values followed by different letters in the same line are significantly different ($P < 0.05$)

Analysis of the content of the metals in the shells, hepatopancreas, and foot tissues of the snails that came from one habitat and had shells with one band on a yellow, pale brown, or pink background, revealed relatively high variation depending on the element and on the type of tissue (Tab. 3).

It should be noted that the concentration and accumulation of iron, lead, and zinc exhibited the same tendency both in the shells and in the hepatopancreas and foot tissues of snails of all three colour types. The most iron was noted in the shells and soft tissues of snails with pale brown shells, and the least in those with pink shells. The most lead was observed in the bodies of pink-shelled snails, and the least in those with yellow shells. The most zinc was accumulated by snails with pink shells, and the least in those with pale brown shells. In the case of zinc and lead, the accumulation capacity of the hepatopancreas is proportionally many times greater than that of foot tissues. On the other hand, biotransformation of iron, its availability and assimilability, and its incorporation into snail tissues is markedly different than in the case of the other two metals. The differences in iron concentration between the tissues tested are not so great as in the case of lead and zinc (Tab. 3).

Table 3. Content of metals in the bodies of *Cepaea nemoralis* snails (n = 10) with different shell colours with one band from habitat S4 (μg/g DM)

Sample	Y 00300	PB 00300	P 00300
SH-Fe	318.56 ±4.27^{ab}	325.98 ±5.98^{b}	312.34 ±3.09^{a}
H-Fe	390.85 ±7.08^{ab}	420.13 ±9.01^{b}	371.07 ±6.44^{a}
F-Fe	213.55 ±2.11^{ab}	280.12 ±2.76^{b}	197.27 ±1.76^{a}
SH-Pb	0.079 ±0.002^{a}	0.087 ±0.006^{ab}	0.099 ±0.006^{b}
H-Pb	0.225 ±0.008^{a}	0.264 ±0.010^{b}	0.337 ±0.013^{c}
F-Pb	0.009 ±0.001^{a}	0.020 ±0.002^{b}	0.035 ±0.002^{c}
SH-Zn	139.12 ±3.99^{b}	102.21 ±3.41^{a}	155.02 ±4.67^{c}
H-Zn	1596.95 ±17.02^{b}	1263.84 ±15.67^{a}	1723.41 ±20.98^{c}
F-Zn	154.05 ±4.01^{ab}	134.18 ±2.54^{a}	215.23 ±6.75^{c}

Y – yellow, PB – pale brown, P – pink, SH – shell, H – hepatopancreas, F – foot tissues
Values followed by different letters in the same line are significantly different ($P < 0.05$)

In very low concentrations, heavy metals are of importance in metabolic processes in animals and plants [Menta and Parisi 2001], but when levels of exposure and accumulation are high, their accumulation in organisms can have toxic effects at various organizational levels, i.e at the molecular level in transcription and translation processes, and at the cytogenetic, cellular, tissue, and organ levels.

CONCLUSIONS

Differences were observed in the amount of accumulated iron, lead, and zinc in the selected morphic types of *Cepaea nemoralis*, and the tendency of changes in metal content in different body parts of the types selected was the same, so it may be suggested that the metals analysed may potentially affect the regulation of expression of the traits responsible for pigmentation.

Bioaccumulation of metals in the shells is long-term and fairly permanent, while toxic agents that are harmful or occur in excess can be easily eliminated from soft tissues. Hence the search for biomarkers of exposition by determining metal accumulation in tissues should take into account which organs and tissues will provide more significant and reliable data.

Many authors are inclined to recognize the role of chance elements in expression of polymorphism. This can be described as a „scientifically safe" approach, as these factors can include most of those mentioned above, including an unlimited number of environmental parameters.

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WYKORZYSTANIE WYBRANYCH ASPEKTÓW POLIMORFIZMU *Cepaea nemoralis* (Gastropoda: Pulmonata) W BIOMONITORINGU ŚRODOWISKA NA TERENIE LUBLINA

Streszczenie. Badano wielkość poziomu żelaza ołowiu i cynku w różnych tkankach wstężyka gajowego – *Cepaea nemoralis* oraz w próbach roślin i ziemi pochodzących z sześciu siedlisk o różnym stopniu antropopresji na terenie Lublina. Oznaczono 28 form barwnych wskazujących na duże zróżnicowanie polimorficzne badanego taksonu na wytypowanych stanowiskach. Analiza zawartości metali w częściach ciała różnych typów barwnych sugeruje potencjalny wpływ analizowanych metali na regulację ekspresji cech fenotypowych i potwierdza użyteczność gatunku w poszukiwaniu biomarkerów ekspozycji.

Słowa kluczowe: *Cepaea nemoralis*, polimorfizm, metale ciężkie, bioakumulacja, monitoring środowiska, biowskaźnik