Measures of the developmental stability, body size and body condition in the black-striped mouse (*Apodemus agrarius*) as indicators of a disturbed environment in northern Serbia

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ABSTRACT. In the present study, an additional combination of end-points was used to evaluate the effects of industrial development on the natural population of the black-striped mouse (*Apodemus agrarius*), previously estimated using cytogenetic and morphometric assays. Developmental stability was assessed by determining the level of fluctuating asymmetry (FA) and the total amount of phenotypic variability (PV). Body weight (BW) and body length (BL) were also used as indices of body size, while the quotient between observed and expected values of body weight presented an index of body condition (BCI). The experimental design employed in this study is unique because only a few studies have been conducted with *A. agrarius* and because no investigator has previously used this combination of end-points in an environmental quality monitoring study. FA was increased in the polluted area for foramen parietalis, foramen dentale, foramen palatinum and foramen angularis. Males from the polluted site had significantly higher FA for foramen parietalis compared with male mice from the reference area. Juvenile animals from the polluted area had significantly higher FAs for foramen dentale and foramen palatinum compared to adults. However, when all the foramina were considered together, a three-way analysis of variance revealed that there was no significant interaction between the factors (sex x age category x site) on the FA (average value). A comparison of PVs indicated higher values in the polluted area for all analyzed characters. For foramen parietalis and foramen dentale, PVs were significantly greater in the polluted site than in the control area. Within the same sex, PVs were significantly greater in mice from the polluted site compared with mice from the control area. The results also indicated that mice trapped at the contaminated site had a reduced body size and poorer body condition. Adult mice exhibited better body condition than juveniles, as revealed by the significantly higher BCI values. Finally, to investigate the relative importance of the three potential biomarkers, a multivariate analysis of variance (MANOVA procedure) was performed. The MANOVA's results revealed the significant effects of age category, site and interaction for sex x site on FA (average value), size and body condition. In conclusion, the results indicated that despite *A. agrarius's* high tolerance to contaminants it may be an important species for environmental quality evaluation studies.

KEY WORDS : body size, body condition, developmental stability, fluctuating asymmetry, foramina.

INTRODUCTION

Typically, the processes of evaluating effects of polluted environments on natural populations of small mammals have been based on a single type of end-point (e.g. cytogenetic, morphometric or morpho-physiological techniques).

Mice from the genus *Apodemus* have been shown to be relevant pollution bioindicators (BERRY, 1975; ABRAMSSON-ZETTERBERG et al., 1997; STOPKA & MCDONALD, 1999; ADRIAN et al., 2002; DAMEK-POPRAWA, 2003; IEARDI et al., 2003; METCHEVA et al., 2001; TOPASHKA-ANCHEVA et al., 2003). The consequences of such exposure on the biology of animals have been assessed using cytogenetic assays, such as: micronuclei frequencies (ABRAMSSON-ZETTERBERG et al., 1997; IEARDI et al., 2003), chromosomal aberrations frequencies (METCHEVA et al., 2001; TOPASHKA-ANCHEVA et al., 2003; VELICKOVIC, 2004) and sperm abnormalities (IEARDI et al., 2003) or morpho-physiological assays, such as: morphological (ADRIAN et al., 2002; DAMEK-POPRAWA, 2003), morphometric (BERRY, 1975; NUNES et al., 2001b; STOPKA & MCDONALD, 1999; VELICKOVIC, 2004) and hematological parameters (NUNES et al., 2001a; TOPASHKA-ANCHEVA et al., 2003).

In the present study an additional combination of end-points was used to evaluate the effects of industrial development on the natural population of the black-striped mouse (*Apodemus agrarius,* Pallas 1771) previously estimated using cytogenetic and morphometric assays (VELICKOVIC, 2004). Here, besides measuring developmental stability (DS), I also examined the consequences of disturbed environment on body size and body condition.

Developmental stability (DS), (MATHER, 1953; THODAY, 1955; or "developmental homeostasis" LERNER, 1954) refers to the ability of an individual to produce a consistent phenotype in a given environment (GRAHAM et al., 1993). Reduced DS can result from wide variety of environmental and/or genetic perturbations (VALENTINE & SOULÉ, 1973; VALENTINE et al., 1973; SIEGEL & DOYLE, 1975a; 1975b; 1975c; SIEGEL et al., 1992; YABLOKOV, 1986; CLARKE, 1992; 1993).

Fluctuating asymmetry (FA), directional asymmetry (DA) and antisymmetry (AS) are the three recognized types of asymmetry in morphological traits. Each is characterized by a different combination of the mean and variance of the distribution of (R & L) differences (VAN VALEN, 1962; PALMER & STROBECK, 1986; PALMER, 1994), where R and L represent the measurement of the right and left sides of bilaterally symmetrical traits respectively.

FA is characterized by normally distributed differences about a mean of zero (Van Valen, 1962; Leary & Allendorf, 1989; Parsons, 1990). Moller & Swaddle (1997) indicated that the metrics for conducting fluctuating asymmetry analysis are derived from taking the difference between right and left traits of bilaterally symmetric organisms and that “they are simple and straightforward to measure, and can be statistically very powerful at detecting differences among populations”.

DA is characterized by a normally distributed (R-L) where the mean departs significantly from zero. AS is associated with a bimodal distribution of (R-L) about a mean of zero or more subtly as a broad peaked unimodal (platykurtic) distribution (Palmer & Strobeck, 1986).

Fluctuating asymmetry (Van Valen, 1962), where differences in the development of the two sides of a bilaterally symmetrical character are random (Palmer and Strobeck, 1986), has been proposed as an indicator of environmental as well as genetic stress (Leary & Allendorf, 1989; Clarke, 1992; Parsons, 1992; Markow, 1995). Stress is considered to be a significant and lasting deviation from favourable conditions that leads to abnormal demands and the destabilization of vital processes (Larcher, 2000).

Furthermore, FA has been extensively used as a measure of developmental instability (DI, the inability of a bilateral organ or organism to buffer its development against disturbances and to produce a predetermined phenotype), Møller & Swaddle (1997). DI, measured as FA, is expected to be positively related to stress and negatively to fitness. Interestingly, the level of fluctuating asymmetry in morphometric characters has been successfully used as a measure of DI in house mouse (*Mus musculus*, Linnaeus 1758) hybrid zones (Alibert et al., 1994; Alibert et al., 1997; Chatti et al., 1999).

The total amount of phenotypic variability (PV), (Soulé, 1982; Pankakoski et al., 1987; Zakharov, 1987) can be used as a measure of DI when changes in developmental “noise” are responsible for changes in the total phenotypic variability of the population (Pankakoski et al., 1987). According to Hallgrimsson et al. (2002), developmental stability and canalization (the ability of a structure to develop along an ideal developmental trajectory under a variety of different environmental conditions; Waddington, 1940) are patterns of phenotypic variability. “Both DS and canalization reflect the tendency for development to follow a preferred trajectory toward some phenotypic outcome”, Hallgrýmsson et al. (2002).

Several studies have shown that animal species display developmental instability (DI) under various stress conditions, reflected in increased FA and PV, observed in laboratory experiments as well as in natural populations (Bailit et al., 1970; Valentine & Soulé, 1973; Valentine et al., 1973; Ames et al., 1979; Siegel & Doyle, 1975a; Siegel & Doyle, 1975b; Siegel & Doyle, 1975c; Zakharov, 1981; Zakharov, 1984; Pankakoski et al., 1987; Zakharov et al., 1991; Stub et al, 2004; Sørensen et al., 2005). Increased environmental stress can therefore be expected to lead to an increased breakdown of homeostatic mechanisms, resulting in increased FA and phenotypic variability (Vøllestad et al., 1998).

Body size is the most obvious and fundamental characteristic of an organism and accordingly has long been a subject of interest (Smith et al., 2004). Previous studies have shown that measuring body size is an effective way to estimate the effects of exposure to environmental pollutants on wild animals. In mammals, reduced body size due to environmental stress has been extensively documented in the laboratory, but more seldom in the wild (Palmer & Strobeck, 1986; Pankakoski et al., 1987; Alcántará & Diaz, 1996; Cavallini, 1996; Cristoffer, 1991; Nunes et al., 2001a; Radwan, 2003).

Additionally, many authors assume that animals that are heavier than predicted by their body size have more metabolizable tissue than individuals that are lighter than predicted by body size (Dobson, 1992); it is unlikely that this extra mass is composed strictly of fat. Unless animals are depositing energy (fat) for a specific purpose such as migration or hibernation, it seems likely that the variation in condition reflects the variation in all constituents of body composition, including fat, protein, water and skeletal tissue (Schulte-Hostedde, 2005).

An animal’s body condition refers to its energetic state, so an animal in good condition has higher energy reserves (usually fat) than an animal in poor condition (Schulte-Hostedde et al., 2001; Schulte-Hostedde, 2005). In mammals, the amount of fat that an individual carries can have important fitness consequences (Schulte-Hostedde et al., 2001). For instance, individuals with larger fat reserves may have better fasting endurance and a higher survival rate than individuals with smaller reserves (Millar & Hickling, 1990). On the other hand, locomotion and predator avoidance can be compromised by heavy fat reserves (Trombulak, 1989). Still, measuring the body condition of live animals has been the subject of much recent debate (Jakob et al., 1996; Kotiaho, 1999; Green, 2001; Hayes & Snokwiler, 2001; Schulte-Hostedde et al., 2001; Speakman, 2001).

The study tested the hypothesis that mice from a polluted area 1) would exhibit a lower developmental stability (DS), 2) would have reduced body sizes and 3) would have an inferior body condition compared to mice from an unpolluted reference site. To test this hypothesis, developmental stability was assessed by determining the level of fluctuating asymmetry and the total amount of phenotypic variability. Body weight and length were also used as indices of body size, while an index of body condition presented the measure of body condition in *Apodemus agrarius.*

MATERIALS AND METHODS

Study areas

This study was performed at two areas in northern Serbia. The contaminated site (Pancevo) is the site of a large petrochemical and fuel storage complex. It is located approximately 15km northeast of the capital (Beograd) and includes an ammonia plant (founded in 1962), a factory for chemical fertilizers (founded in 1975), and a crude oil refinery (founded in 1968). Here it is necessary to indicate certain chemical compounds, their metabolites and unwanted by-products, because they represent some of the most dangerous chemical pollutants producing long-term negative effects on the environment, human health and living organisms. About 700 tons of liquid ammonia (NH_3) and 500 tons of urea (CH_4N_2O) are processed daily, and about $8x10^3$ tons of hydrochloric acid (HCl, 33%), 109,600 tons of vinyl chloride monomer (VCM, C_2H_3Cl), $200x10^3$ tons of ethylene dichloride ($C_2H_4Cl_{12}$), $4850x10^3$ tons of crude oil products are produced annually. At the petrochemical plant, chlorinated solvents such as trichloromethane, tetrachloromethane, trichloroethane, dichloroethene, trichloroethene, and others closely associated with the unwanted by-products of PVC (polyvinyl chloride) production, were found in both soil and groundwater samples (Gopal & Deller, 2002). Additionally, VCM is a human carcinogen known to cause liver and blood tumors. VCM released into the atmosphere is protected from polymerization and remains dangerous for a long period for the environment and to humans. The combustion of VCM generates cyclic dioxins that are internationally recognized cancer inducers. Soot is extremely carcinogenic; its toxicity is attributable to high concentrations of polycyclic aromatic hydrocarbons; they are metabolically transformed by aquatic and terrestrial organisms are into carcinogenic and mutagenic metabolites.

The reference site (Cer), a forested area, is located 150km to the west of the polluted site, far from any known contamination.

Sampling and statistical treatment

From 1994-2000 (except in 1999), mice were trapped live using Longworth traps baited with mixture of sardines and grain. One hundred traps were set in the morning at intervals of approximately 5m, along 500m transects. The animals were collected the following morning.

In the laboratory, the mice were sacrificed and analyzed within three days after capture. All analyses (developmental stability, body size and body condition) were performed using the same individuals.

A total of 156 *Apodemus agrarius* were studied: 68 from the polluted area (34 males and 34 females; 24 juveniles and 44 adults) and 88 from the reference site (60 males and 28 females; 27 juveniles and 61 adults), which were the same used by Velickovic (2004).

Statistical treatment for developmental stability indices

Developmental stability was assessed using quantitative characters presented as a number of paired foramina in the skull. Foramina, the small openings for nerves and blood vessels, were counted macroscopically under a binuclear microscope (16x magnification) on the right and left sides of the cleaned skull without a knowledge of where the animal had been trapped; the skulls were cleaned in a dermestid beetle (*Dermestes maculatus*) colony.

The abbreviations for foramina are (Fig. 1): *foramen parietalis* (F_1), *foramen dentale* (F_2), *foramen palatinum* (F_3), *foramen alveolaris maxillaris* (F_4), *foramen mentale* (F_5), *foramen angularis* (F_6).

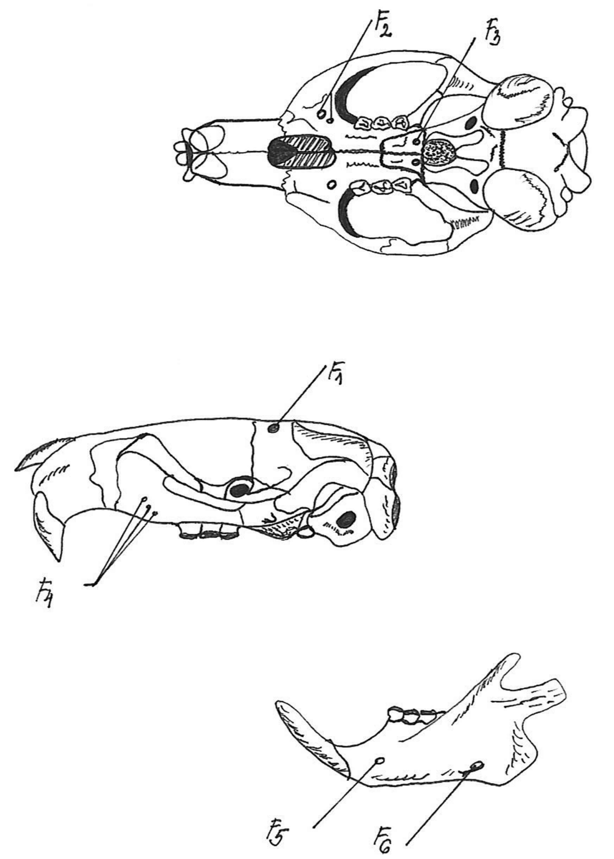

Fig. 1. – Skull foramina used in the study. See the text for abbreviation references.

This set of meristic (non-metric) characters was selected because it has been shown that meristic characters are amenable to analysis using quantitative genetic methods (Falconer, 1989) and because developmental stability (measured as FA) has been assessed using the numbers of paired foramina in the skull (Berry, 1969; Zakharov & Yablokov, 1990; Pankakoski et al., 1992; Pertoldi et al., 1997).

Two indices were calculated separately for each character to describe developmental stability: (1) FA_4: index 4 of Palmer & Strobeck (1986), Palmer (1994) and (2) the total phenotypic variability of foramen numbers (PV), Zakharov (1984).

(1) $FA_{4\ (foramina)} = Var\ (R_i - L_i)$

(2) $PV_{\ (foramina)} = Var\ (r_i + l_i)$,

where $(R_i - L_i)$ = signed asymmetry, R_i and r_i = value of the character on the right side of the skull, L_i and l_i = value of the character on the left side of the skull, Var = variance.

Before proceeding with the asymmetry analysis, statistical tests were carried out to detect the features confounding the analyses of FA (Palmer & Strobeck, 1986; Palmer, 1994) such as: directional asymmetry (DA) and antisymmetry (AS).

Firstly, the deviation from the normality of the (R_i -L_i) distributions was assessed using the Kolmogorov-Smirnov test of normality. These distributions were tested for significant skewness (g_1) and kurtosis (g_2) according to SOKAL & ROHLF (1981). Secondly, a one-sample *t*-test for a departure of the mean of (R_i - L_i) from an expected mean of zero was performed. Thirdly, for each character within samples a simple linear regression of absolute asymmetry $|(R_i - L_i)|$ on (R_i + L_i)/2 was assessed.

Because the FA and PV values are variances, statistical comparisons between samples can be detected by comparing the heterogeneity of variances. Although this was implemented by performing the *F*-test, a sequential Bonferonni correction (RICE, 1989) was applied to avoid "false" significant results. The Friedman test was used to compare the medians of variances.

Statistical treatment for body size and body condition measures

For each mouse, the measurements of body size were recorded as body length (BL) calculated by subtracting the tail length from the total length (1mm accuracy) and body weight (BW) measured to the nearest 0.1mg (embryo weights were deducted from the body weight of pregnant females). The expected weight for a given length was obtained from a linear equation of the logarithms of BW and BL (in males and females, using an analysis of covariance for slopes). Subsequently, an index of body condition (BCI) was calculated for each mouse as the quotient between observed and expected values of BW, (NUNES et al., 2001a).

All these variables were checked for normal distribution using the Kolmogorov-Smirnov test of normality.

The analyses of the main effects of site, sex, age category and all possible interactions of these variables on BW, BL and BCI were performed using three-way ANOVA (analysis of variance) procedures.

RESULTS

Preliminary results of asymmetry analyses

For the three (R_i-L_i) distributions considered in this study, normality was rejected: (in Cer: *foramen mentale* d=0.374, P<0.01; in Pancevo: *foramen mentale* d=0.371, P<0.01 and *foramen angularis* d=0.165, P<0.05).

The results of the regression analyses between $|R_i-L_i|$ and (R_i+L_i)/2 were significant: at Cer (for *foramen alveolaris maxillaris*, r=0.400, P=0.000; for *foramen mentale*, r=0.747, P=0.000 and for *foramen angularis*, r=0.260, P=0.016) and at the Pancevo site (for *foramen parietalis* r=0.830, P=0.000; for *foramen dentale* r=0.677, P=0.000; for *foramen palatinum* r=0.273, P=0.027; for *foramen alveolaris maxillaris* r=0.399, P=0.001, and for *foramen mentale* r=0.628, P=0.000).

For cases of significant linear regression of absolute asymmetry $|(R_i - L_i)|$ on (R_i + L_i)/2 value, the index chosen to measure FA (FA_4) was recalculated as the value of (R_i-L_i) divided by (R_i+L_i)/2, index 6 (FA_6 of PALMER & STROBECK, 1986; PALMER, 1994)

$$FA_{6\ (foramina)} = Var\,[(R_i-L_i)/\ ((R_i+L_i)/2)]$$

where (R_i - L_i) = signed asymmetry, R_i = value of the character on the right side of the skull, L_i = value of the character on the left side of the skull, Var = variance.

To ensure the corrections' successful implementation, I again correlated the corrected (R_i-L_i) values [(R_i-L_i)/(R_i+L_i)] with ((R_i+L_i)/2).

The results of the *t*-test showed that there were no significant departure of the mean of (R_i-L_i) from the expected mean of zero in both samples (no DA asymmetry is present, all P>0.05). Three out of twelve (R_i-L_i) distributions were significantly skewed (two in Cer: for *foramen parietalis*, g_1=-35.671, P<0.01, and for *foramen angularis* g_1=2.145, P<0.05, and one in Pancevo: for *foramen parietalis* g_1=12.445, P<0.01). A significant platikurtic or bimodal distribution (-g_2 value) was not found; thus no antisymmetry is present.

Developmental stability indices

Higher values of FA were found in the polluted area for all characters except for *foramen alveolaris maxillaris.* The *F*-test results also showed that in five out of six characters the variances describing FA differ significantly between the two areas (P_r=0.05). Specifically, for four out of these five characters (*foramen parietalis*, *foramen dentale*, *foramen palatinum*, and *foramen angularis*), FAs were higher (developmentally less stable) in the contaminated area than in the reference area. For *foramen mentale* however, FA was significantly higher in the control area, Table 1.

TABLE 1

Comparison of fluctuating asymmetry (Asymmetry) and total phenotypic variability (Variability) in foramen numbers between the polluted (Pancevo=$P_{AN.}$) and control (Cer=C) areas.

Foramen	Asymmetry		P_r	Variability		P-value
	C (N)	$P_{AN.}$ (N)		C (N)	$P_{AN.}$ (N)	
F_1	0.112 (86)	0.225 (67)	*	0.012 (87)	0.371 (67)	**
F_2	0.558 (85)	1.108 (68)	*	0.856 (87)	1.340 (68)	**
F_3	0.304 (85)	0.623 (66)	*	38.854 (86)	53.421 (66)	NS
F_4	0.459 (86)	0.286 (67)	*	16.001 (86)	17.865 (67)	NS
F_5	0.128 (86)	0.125 (68)	NS	0.304 (86)	0.362 (68)	NS
F_6	0.263 (86)	4.682 (68)	*	16.327 (86)	16.355 (68)	NS

N =sample size, NS =statistically not significant, Pr =revised probability values (results of the sequental Bonferonni correction), * = significant at P<0.05, ** = significant at P<0.01

Moreover, the results obtained results demonstrated significant differences in FA levels between sites within the same sex (males from the polluted area had a significantly higher FA value for *foramen parietalis*; F=5.547, P=0.025).

Concerning age categories, a statistically significant difference in FA levels was observed between juvenile and adult mice from the polluted area. Juvenile animals had significantly higher FAs for two characters ($F_{foramen\ dentale\ (23,26)}$ =9.313, P<0.05, and $F_{foramen\ palatinum\ (23,26)}$ =7.653, P<0.05) compared to adults from the same site.

However, when all the foramina were considered together, a three-way analysis of variance revealed that there was no significant interaction between the factors (sex x age category x site) on the FA (average value), Table 2. The average FA value in foramen numbers was calculated by dividing the sum of FAs by the total number of characters analyzed.

TABLE 2

Results of the three-way analysis of variance relating the FA (average value) with age category, sex and site.

Source of variation	df	MS	F-ratio	P-value
Age category	1	0.059	0.114	0.736 NS
Sex	1	0.004	0.008	0.930 NS
Site	1	0.586	1.121	0.291 NS
Age category x Sex	1	0.688	1.316	0.253 NS
Age category x Site	1	0.037	0.070	0.791 NS
Sex x Site	1	0.987	1.887	0.172 NS
Age category x Sex x Site	1	0.007	0.014	0.908 NS
Error	137	0.523		

NS = statistically not significant

PV values differ at the investigated sites; a comparison of PVs indicated higher values in the polluted area for all analyzed characters. For two out of six characters (*foramen parietalis* and *foramen dentale*), the PV values were significantly higher in the polluted area than in the reference area, Table 1.

When sexes were separated and tested for PV, the results indicated a difference between the sites within sexes. Within both sexes, the PVs were significantly higher in mice from the polluted area compared with mice from the unpolluted site (for males: $F_{foramen\ parietalis}$ =7.354, P=0.008, and for females: $F_{foramen\ dentale}$ =4.524, P=0.038).

When all the foramina were considered together, the FA does not differ significantly between the two areas, although the PV was significantly higher at the contaminated site (Friedman's test results for median values, P<0.014), Fig. 2.

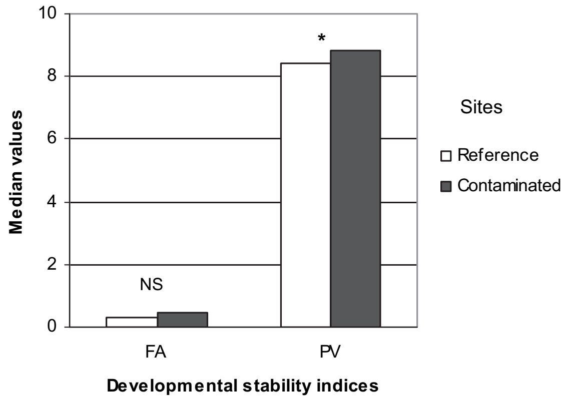

Fig. 2. – Comparison of developmental stability indices (fluctuating asymmetry = FA and total phenotypic variability = PV) in *A. agrarius* in the polluted and control areas. The values on the y-axis are medians (FA; PV), NS= statistically not significant, * = statistically significant at P<0.014.

Body size and body condition

Body length, body weight and an index of body condition produced normal distributions at both sampling sites. However when each sex was analyzed separately, normality was rejected for the females from the control area (for body length, P<0.05) and for males from the polluted area (for body weight, P<0.05).

Results also indicated that (1) the coefficients of variation (CVs) for body size measures were lower in mice from the polluted area compared to mice from the reference site except for BL and (2) CVs were largest for body weight and the smallest for body length within the sites, Table 3.

When CVs were computed by age category, site and sex, the highest value was detected for the index of body condition in adult males from the reference site (CV=19.719), while the smallest value exists for body length in juvenile males from the polluted area (CV=3.351).

TABLE 3

Descriptive statistics for body measurements in Cer (reference) and Pancevo (contaminated) sites

Site	Variable	N	Mean ± S.D.	Min.	Max.	CV
Cer	BW (g)	88	18.717±4.420	11.100	32.703	23.605
	BL (mm)	89	95.521±6.998	82.000	116.000	7.322
	BCI	88	6.401±1.212	4.082	10.189	18.910
Pancevo	BW (g)	75	17.933±3.725	11.000	30.500	20.800
	BL (mm)	75	92.346±7.836	62.000	116.000	8.494
	BCI	75	6.228±1.030	4.167	9.297	16.530

N = sample size, Mean = mean value, S.D. = standard deviation, Min. = minimum, Max. = maximum, CV = coefficient of variation, BW = body weight, BL= body length, BCI = index of body condition

Linear equations of the logarithms of BW and BL were obtained for each sex separately: for males (y=2.083x–6.555, r^2=0.730, P=0.000) and for females (y=1.597x–4.369, r^2=0.709, P=0.000). The models obtained were highly significant for both sexes (P=0.000). However their expected power is low: 30.1% and 26.8% of the variance explained for males and females, respectively (Fig. 3a and Fig. 3b).

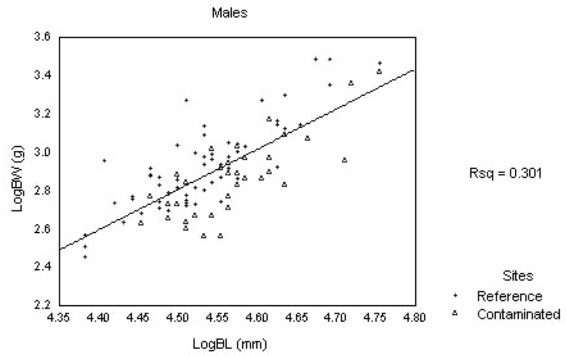

Fig. 3a. – Relationship between body weight (BW) and body length (BL) for male *A. agrarius* specimens.

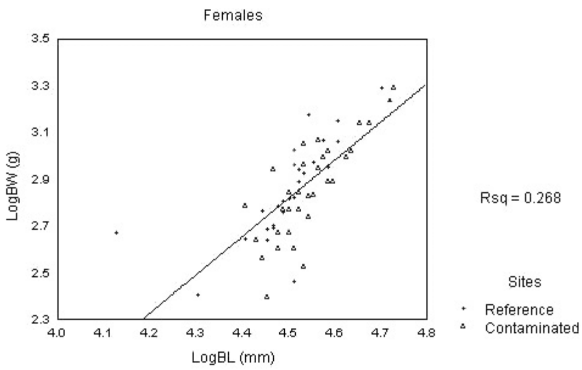

Fig. 3b. – Relationship between body weight (BW) and body length (BL) for female *A. agrarius* specimens.

The effects of sex, age category, site and interactions of these variables on BW, BL and BCI were assessed by using the ANOVAs (Table 4). No effects of possible interactions of sex, age category and site on BW, BL and BCI values were observed. Significant differences were however detected in BW between age categories: ($19.665_{(g)}$ vs. $15.574_{(g)}$, for adults and juvenile individuals, respectively; F= 45.686, P=0.000).

In BL, significant differences were observed between sexes: ($94.623_{(mm)}$ vs. $92.677_{(mm)}$, for males and females respectively; F=4.465, P=0.040), between age categories ($96.286_{(mm)}$ vs. $88.717_{(mm)}$, for adults and juvenile individuals, respectively; F=48.268, P=0.000) and between sites ($95.520_{(mm)}$ vs. $92.348_{(mm)}$, in the control area and the polluted area, respectively; F=16.770, P=0.000).

BCI in adults was significantly higher than in juveniles (6.663 vs. 5.589; F=38.783, P=0.000) and in mice from the reference site than in the polluted area (6.403 vs. 6.234; F=5.757, P=0.020).

TABLE 4

Table 4. Results of the three-way ANOVAs relating BW (body weight), BL (body length) and index of body condition (BCI) with sex, age category, site and all their possible interactions

Variable	Effects of interactions	F - ratio	P - value
BW	Sex (1)	0.099	0.753
	Age category (2)	45.694	0.000*
	Site (3)	1.084	0.299
	1x2	0.014	0.905
	1x3	2.547	0.112
	2x3	0.070	0.791
	1x2x3	0.081	0.775
BL	Sex (1)	4.471	0.036*
	Age category (2)	48.270	0.000*
	Site (3)	16.772	0.000*
	1x2	0.495	0.482
	1x3	0.851	0.357
	2x3	2.933	0.088
	1x2x3	0.059	0.807
BCI	Sex (1)	0.054	0.815
	Age category (2)	38.781	0.000*
	Site (3)	5.761	0.017*
	1x2	0.132	0.716
	1x3	2.481	0.117
	2x3	0.796	0.373
	1x2x3	0.207	0.650

* = statistically significant at P<0.05

Finally, to investigate the relative importance of the three potential biomarkers, a multivariate analysis of variance (MANOVA procedure) was performed. The MANOVA's results revealed the significant main effects of age category and site on FA (average value), size and body condition, Table 5. The MANOVA's results also showed significant interaction for sex x site. Significant test results for the MANOVA procedure were based on *F* statistics derived from Wilks' lambda.

TABLE 5

MANOVA`s results for the effects of sex, age category, site and their interaction on FA, body size and body condition values in *Apodemus agrarius*.

Source of variation	$df_{(1,2)}$	Wilks`s lambda	P - value
Sex	3,141	0.974	0.332
Age category	3,141	0.728	1.84×10^{-9}***
Site	3,141	0.738	4.97×10^{-9}***
Sex x Age category	3,141	0.990	0.489
Sex x Site	3,141	0.950	0.028*
Age category x Site	3,141	0.982	0.277
Sex x Age category x Site	3,141	0.996	0.742

*=P<0.05; ***=P<0.001

DISCUSSION

Assessing the impacts of environmentally-generated stress factors on natural plant and animal populations is a complex task because organisms in the environment are exposed to a diverse range of uncontrolled variables such as weather conditions, parasites, mixtures of pollutants). Within the environment, the synergistic and antagonistic effects of these factors are not always fully understood. Additionally, the presence of environmental pollutants in small mammals is relatively easy to analyze, but it is difficult to measure their effects on population parameters. In fact, VALENTINE et al. (1973) and PARSONS (1992) concurred that pollutants must reach substantial concentration levels before they observably affect population density, viability and breeding success. However, a positive benefit of *in situ* environmental quality monitoring studies is that the obtained results can tell us what really happens in the environment. The experimental design employed in this study is unique because only a few studies have been conducted with *A. agrarius* and because no investigator has previously used this combination of endpoints in an environmental quality monitoring study.

Several researchers have examined the variability in the numbers of paired foramina as an expression of developmental stability (measured as FA) and have found differences between natural populations (BERRY, 1969; ZAKHAROV & YABLOKOV, 1990; PANKAKOSKI et al., 1992). Specifically, these authors found that a disruption in developmental stability was observed in animals affected by different types of environmental pollutants (but see SCHANDORFF, 1997).

The results presented here demonstrated that the level of FA in the polluted area was increased for all foramina except for *foramen alveolaris maxillaries*, and the developmental stability of the same characters decreased.

The fact that FA levels were significantly different between sites within the same sex indicates that it is possible that certain pollutants affect sexes differentially. The study also indicated that the progeny of animals with long-term exposure to environmental pollutants had significantly higher levels of FA for *foramen dentale* and *foramen palatinum*. Thus, the increased FA in mammals seems to be a consequence of stress experienced by mothers and fetuses during pregnancy or lactation (SIEGEL & DOYLE, 1975b) and that the developmental disturbances occurred during the prenatal life or at the early stages of development. On the other hand, the use of energy for stress tolerance that could otherwise be used for developmental control, growth, reproduction, and survival (HOFFMAN & PARSONS, 1989; PARSONS, 1990; ALEKSEEVA et al., 1992; OZERNYUK et al., 1992) impose a distinct developmental stress.

Interestingly, in a 3-way ANOVA correcting for age and/or sex, no effect of site on average FA is found. One of possible explanations would be that there is no replication, and thus by definition the individuals within the two sampling sites are no real replicates.

It is worth remarking that the skulls of rodents have been frequently employed in FA studies; the lower jaw of a mouse has long served as a model system for studying the development and evolution of complex morphological structures (KLINGENBERG, et al., 2003). The measurements that are classically recorded for the mandible (mandible mass, mandible size, maximum width and length of the lower molars, the height and length of the mandible) indicated that increased FA levels in the lower jaw (and associated tooth) dimensions in mice responded to various kinds of stress (SIEGEL & DOYLE, 1975c; SIEGEL et al., 1977; LEAMY, 1984; ATCHLEY et al., 1984; PANKAKOSKI et al., 1992; GILEVA & NOKHRIN, 2001; VELICKOVIC, 2004). However for all of these studies suggesting that stress can increase FA, there are others where no effects, or even the opposite effects, of stress on FA have been observed (LEAMY et al., 1999a; MARKOW, 1995; WOODS et al., 1999). Thus, the results obtained in my studies and in other investigations corroborate the potential use of FA on both metrical and meristic characters, as a good diagnostic procedure for assessing stress tolerance. Concurring with this view, LEAMY & KLINGENBERG (2005) suggest that FA may play some kind of role as a fitness indicator.

The data presented here also indicates that both sexes had significantly higher PVs in the polluted area. Interestingly, when all foramina were considered together, the PV value was significantly higher in the polluted area than at the control site. This finding is consistent with similar results in an earlier study (PANKAKOSKI et al., 1992), whose authors revealed that the total phenotypic variance is greater in the common shrew (*Sorex araneus*, Linnaeus 1758) associated with heavy metal pollution than in the reference areas.

The present study therefore indicates that developmental stability is one of the most general characteristics in individual developmental processes and is also a sensitive parameter that can be used for the biomonitoring of natural populations' conditions (SOULÉ, 1967; JONES, 1987; ZAKHAROV, 1981; 1987). An analysis of developmental stability may therefore potentially reveal changes in a population's condition, the changes not yet reflected by disturbances of individual viability (YABLOKOV, 1986). Moreover, by studying developmental stability it might also be possible to assess the synergetic effects of toxic compounds or interactions between pollutants and other stressful factors (ZAKHAROV et al., 1991), which are either complex or impossible to study by other means.

Certain data from the body size and body condition analyses found in this study are similar to findings reported by NUNES et al. (2001a) for the Algerian mouse (*Mus spretus*, Lataste 1883) inhabiting an area contaminated with heavy metals. However, the data from *M. spretus* has been used more as a reference than for direct comparisons. Mice (from both studies) trapped at the contaminated sites have reduced BW as well as BL values. In NUNES et al. (2001a), all analyzed *Mus spretus*, except non-reproductive males, weighed less at the metal-polluted site compared with mice inhabiting the unpolluted area. The reduction of bone lengths and tooth dimensions in laboratory rats raised under effects of various stresses, such as extreme temperatures or noise, has been demonstrated by DOYLE et al. (1977). On the other hand, lead poisoning in laboratory rats results in decreased body weight (GOYER et al., 1970). Thus, the small sizes of mice found in contaminated areas may be explained by the negative effects of chemical pollutants from the environment. However, other causes, such as nutrition quality/availability, cannot be ruled out.

M. spretus and *A. agrarius* mice from the reference areas exhibited better body condition than mice from the polluted sites, revealed by the significantly higher BCI value. Furthermore, the same trend was observed for adults from the investigated localities; compared with juveniles, the adults exhibited better body condition than the juveniles. This observation can be explained as a consequence of normal ontogenetic development; because juveniles reach adult length before they reach adult mass, they will normally go through a period in which they are long relative to their mass. Another possibility may result from the deposition of fat reserves during the breeding season (Millar, 1975).

The MANOVA's results revealed the significant effects of age category, site and interaction for sex x site on FA (average value), size and body condition.

Conclusions and prospects for future studies

The present study indicated that developmental stability indices, combined with body size measures as well as an index of body condition can be used to detect, analyze and evaluate the effects of environmentally-generated stress factors on natural populations of small mammals. Therefore, I propose the use of this combination of endpoints as an ecotoxicological biomarker in pollution monitoring.

In conclusion, the results obtained from previous researches and the present study demonstrated that despite *A. agrarius's* high tolerance to contaminants (Petrov, 1992), it may be an important species for environmental quality monitoring.

For future work, it would be interesting to compare the results of investigations performed on *Apodemus agrarius* with new data obtained from other species of the genus *Apodemus* and/or other species of small mammals indigenous to Serbia.

However, it would be important to consider the following: 1) to increase number of investigated localities in order to avoid pseudoreplication. According to Hurlbert (1984) pseudoreplication is defined as the use of inferential statistics to test for treatment effects with data from experiments where either treatments are not replicated (though samples may be) or replicates are not statistically independent 2) that each polluted site should be scored for quantitative variables such as types and concentrations of environmental pollutants 3) a larger sample size is necessary because increases statistical power (Cohen, 1988; Yezerinac et al., 1992).

ACKNOWLEDGEMENTS

I thank Marija Velickovic, Bratislav Toskovic and three anonymous reviewers for their more than valuable comments on an earlier version of this manuscript. Roger Freundlich is gratefully acknowledged for correcting the English text. Also, I thank Slavica S. Borkovic for technical help. The Ministry of Science and Environmental Protection of the Republic of Serbia supported this work.

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Received: July 27, 2005
Accepted: March 30, 2007