THE EFFECT OF LEAD ON THE DEVELOPMENTAL STABILITY OF *DROSOPHILA SUBOBSCURA* THROUGH SELECTION IN LABORATORY CONDITIONS

ZORANA KURBALIJA[1], B. KENIG[1], JOVANA PLAVŠA[3],
MARINA STAMENKOVIĆ-RADAK[1,2] and M. ANĐELKOVIĆ [1,2]

[1] *Institute of Biological Research, University of Belgrade,* 11000 Belgrade, Serbia
[2] *Faculty of Biology, University of Belgrade,* 11000 Belgrade, Serbia
[3] *Faculty of Science, University of Novi Sad,* 21000 Novi Sad, Serbia

Abstract – Fluctuating asymmetry (FA), the increased variation of bilateral symmetry in a sample of individuals, can indicate disturbance in developmental stability caused by environmental and/or genomic stress. This developmental instability was analyzed in *Drosophila subobscura* maintained for seven generations on two different concentrations of lead in laboratory conditions. The FA4 index showed that the genotypes reared on the higher lead concentration were in developmental homeostasis, except for males in the F7 generation, for both wing size parameters. The results show that different degrees of lead pollution cause different responses to selection of the exposed population in laboratory conditions.

Key words: Developmental instability, fluctuating asymmetry, lead pollution, *Drosophila*

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INTRODUCTION

Environmental changes caused by anthropogenic activities expose many wild plant and animal populations to increasing levels of environmental stress, and to increased risk of their extinction. Concerns about the ability of organisms to adapt to anthropogenic forms of stress have grown steadily (Bijlsma and Loeschcke, 1997; Hoffmann and Parsons, 1997; Forbes, 1999; Walther et al. 2002; Frankham, 2005). Heavy metal pollution, either naturally occurring or man-produced, is regarded as the most important stress agent (Lindgren & Laurila, 2005; Sørensen et al., 2005; Sabovljevic et al., 2007) and has become a significant environmental problem in industrialized countries, with lead as the most widespread pollutant (Nriagu and Pacyna, 1988; Grubor, 2008).

Natural and laboratory populations have been shown to persist under stressful conditions, suggesting that environmental stress may cause divergence in traits related to stress resistance among differently exposed populations (Bijlsma and Loeschcke, 1997; 2005.). Such small-scale evolutionary processes might lead to alterations in the genetic architecture of populations and, eventually, to genetic erosion, not only in traits directly related to stress resistance, but also in traits that are genetically correlated with stress resistance (Perez and Garcıa, 2002; Van Straalen & Timmermans, 2002; Merila et al., 2004).

The evolution of heavy metal tolerance has been well documented in natural populations of plants (Antonovics et al., 1971; Macnair, 1993; Shaw, 1999; Pauwels et al., 2006), aquatic invertebrates (Klerks and Weiss, 1987; Martinez and Levinton, 1996), and terrestrial invertebrates (Posthuma and Van Straalen, 1993; Shirley and Sibly, 1999; Morgan et al., 2007). Diptera, and in particular Drosophila, are favorable model organisms in studies of heavy metal resistance, a widespread phenomenon in invertebrates. Exposure to metal compounds such as cadmium, lead, copper, zinc, or mercury give rise to genetic responses in Drosophila melanogaster (Chapco et al. 1978; Magnuson et al.

1986; Gill et al. 1989; Maroniet et al. 1985; Shirley et al. 1999) and the development of flies depends on heavy metal concentrations and the number of generations of exposure.

Developmental instability (DI), as an indicator of environmental stress, refers to an individual inability to produce a specific phenotype under a given set of conditions. A number of studies show that DI is positively associated with the level of stress experienced by an individual (Palmer, 1994; Lens et al., 2002).

A common estimator of DI is fluctuating asymmetry (FA), i.e. random deviations from bilateral symmetry, and it has been used in a wide range of studies as a measure of environmental disturbance during an organism's development (Palmer and Strobeck, 1986). There are some data about the utility of FA as a measure of DI. A number of studies report that the asymmetry of bilateral traits increases when organisms develop under stressful conditions (Polak M., 2004; Hoffmann, 2005; Pertoldi et al., 2006; Savic, 2008) because FA often increases with stress (Clarke, 1993; Badyaev et al., 2000), including exposure to toxins and infections with parasites (Clarke and McKenzie, 1987; Polak, 1993). However, there are also contrary results showing lower FA levels under higher stress (Møller, 1997a) and many cases where stressful conditions did not cause changes in asymmetry (Leung and Forbes, 1996; Bjorksten et al. 2000).

Drosophila subobscura (Collin, 1936) is a Palaearctic species which displays a rich inversion polymorphism in all 5 acrocentric chromosomes of the set (Krimbas and Loukas, 1980; Krimbas, 1993). Although the inversion polymorphism of *D. subobscura* is quite stable, it varies with environmental changes, being therefore classified as a semi-rigid or semi-flexible type (Sperlich and Feuerbach, 1966; Andjelkovic et al., 2003). Exposure to various concentrations of lead through several generations had no effect on major parameters of inversion polymorphism of *D. subobscura* (Kalajdzic et al. 2006), although the frequencies of some gene arrangements changed, which is possibly associated with the adaptive processes of evolving heavy metal resistance. There are few data on the effect of lead exposure on fitness components and developmental stability in *Drosophila subobscura* (Stamenkovic-Radak et al., 2008).

The aim of this study was to analyze the direction and range of changes in developmental stability measured by wing fluctuating asymmetry (FA) in males and females of *D.subobscura* flies maintained during seven generations on substrates with different concentrations of lead.

MATERIAL AND METHODS

The F_1 progeny of the wild-caught *D.subobscura* females from the locality *Deliblato sands*, 60 km north-east from Belgrade, Serbia, were used for setting the control and two experimental groups. The control (C) was reared on the standard *Drosophila* medium (water/cornmeal/yeast/sugar/agar/ and nipagine as fungicide) without lead. One experimental group was kept on a lower lead concentration (LLC), with 10 μg/ml of lead acetate ($Pb(CH_3COO)_2 \cdot 3H_2O$) added (0,03 mM solution), and the other experimental group on a higher lead concentration (HLC), with 100 μg/ml of lead acetate added (0,3 mM solution).

All groups were kept in mass cultures, 10 bottles each, containing 25 ml of standard medium, with or without lead acetate. The flies from the F_1 generation were mixed randomly within each group and new mass cultures were used for obtaining the F2 generation. Each subsequent generation (7 in all) was obtained by mixing the parents collected from different bottles within the group. Forty males and females from each group, from the F3, F5 and F7 generations, were frozen (-20°C) and used for wing measurements.

The left and right wings from each fly were cut and prepared on a microscope slide for measurement. The wing length was taken as the distance from the intersection of the third longitudinal vein with the anterior cross vein to the wing tip where the third vein ends. The wing width was taken as the distance between the ends of the second and the fifth longitudinal vein. Measurements were made under a binocular microscope, with a Leica/Cannon Image analysis system.

Fluctuating asymmetry analysis

Fluctuating asymmetry is a pattern of bilateral variations in a sample of individuals, where the mean of (R-L) is zero and the variation is normally distributed around that mean. In the present study we used the two most common FA indices, FA1 and FA4 (Palmer, 2001). The FA1 index of each trait was measured as the absolute (unsigned) |R-L| difference between the samples, and the FA4 index was the variance of differences between the right and left side (FA4=var(L-D)) (Palmer, 1994).

Before interpreting FA estimates, several statistical procedures were completed. The measurment error was estimated for all samples by the two-way ANOVA on a sample of 30 individuals measured twice (Palmer, 1994). There were significant interactions between the wing size and individual FA for both length (MS=233.06, $p < 0.001$) and width (MS=181.47, $p < 0.001$), which means that FA has a greater value than the measurement error. Non-parametric tests, Shapiro-Wilk, Jarque-Bera, and χ2, were used to test (R-L) for departures from normality. All samples had a normal distribution after a sequential Bonferroni correction. A one-sample *t*-test was done to test the departure of the mean of (R – L) from the expected mean of zero. The signed left-right (L-D) size differences showed that directional asymmetry (DA) is not absent in all samples. To test size dependence on the absolute FA, linear regression analyses of ((R+L)/2) on |R-L| were done for all samples, and the results indicated that FA does not vary with trait size in these samples.

After testing sample distribution, an F-test (Rice, 1989) was performed for differences in variance between the samples, and a t-test for testing the differences in the mean values between the samples. All the statistical analyses were performed using PAST software (Hammer et al., 2001).

RESULTS

The statistics of the mean value ((R+L)/2) of the wing length and width of both sexes in the lead treated groups, in the F3, F5, and F7 generations are shown in Table 1.

A significant difference in wing *length* is obtained between the *males* from the LLC and the HLC (t=-2.22. $p<0.05$) in the F3 generation. A simillar response was found in the F5 generation, and there was a significant difference between the control and the HLC (t=-2.68, $p<0.01$), and between the LLC and the HLC (t = -2.90, $p<0.01$).

The significant difference in the mean value of the wing *width* was found between the males of the control and the HLC (t=-2.25, $p<0.05$), and between the LLC and the HLC (t=-2.47, $p<0.05$) in the F3 generation. The analysis showed a similar pattern in the F5 generation, with a significant difference between the control and the HLC (t=-2.64, $p<0.05$), and between the LLC and the HLC (t=-2.16, $p<0.05$). A significant difference was obtained in the F7 generation between the the control and LLC (t=-2.60, $p<0.05$), and between the control and the HLC (t=-2.21, $p<0.05$).

The statistics of the mean value ((R+L)/2) of the wing *length* of *females* showed a significant difference between the control and the HLC (t=-2.47, $p<0.05$) and between the LLC and the HLC (t=-3.27, $p<0.01$) in the F3 generation. The analysis showed a significant difference between the control group and the LLC (t=-2.85, $p<0.01$), and between the LLC and the HLC (t=2.72, $p<0.01$) in the F7 generation. Also, there was a significant difference in the mean value of the wing *width* between females from the control group and the HLC (t=-3.45, $p<0.001$), and between the LLC and the HLC (t=-3.70, $p<0.001$) in the F3 generation. A significant difference was also found in the F7 generation between the control and the LLC (t=-

Table 1. The mean value of wing length and width in both sexes of *D.subobscura*, for seven generations with and without lead

LENGTH			males				females		
generation	treatment	N	mean(R+L)/2±SE	t test	mean(R-L)±SE	N	mean(R+L)/2±SE	t test	mean(R-L)±SE
F3	C	33	586.42±3.54	1.82	1.01±0.87	33	628.48±4.17	1.19	-1.09±1.28
	LLC	33	577.64±3.26	-0.48	0.09±0.73	31	620.18±5.64	-2.47 *	4.12±1.15
	HLC	33	588.96±3.90	-2.22 *	1.12±0.89	33	643.60±4.49	-3.27 **	1.42±0.93
F5	C	30	588.30±5.75	0.15	0.73±0.86	30	630.53±4.76	0.49	-0.43±1.02
	LLC	31	587.14±5.27	-2.68 **	1.36±0.91	31	627.75±3.05	0.07	1.43±1.05
	HLC	32	613.09±7.15	-2.90 **	3.05±1.12	31	630.01±4.82	-0.40	-1.60±0.81
F7	C	32	567.11±4.93	-1.44	-0.28±0.78	33	617.12±6.20	-2.85 **	-0.67±0.93
	LLC	31	576.01±3.64	-0.61	-3.21±1.14	32	638.49±4.12	-0.72	0.78±0.93
	HLC	33	571.05±4.10	0.90	-2.91±2.17	34	622.48±4.17	2.72 **	-1.46±1.01
WIDTH									
F3	C	33	374.73±2.35	0.01	0.12±0.92	33	404.18±3.02	0.47	0.30±1.45
	LLC	33	374.72±1.75	-2.15 *	1.75±2.25	31	402.01±3.46	-3.45 ***	1.99±1.16
	HLC	33	381.63±2.17	-2.47 *	-0.02±0.93	33	418.63±2.90	-3.70 ***	-1.38±0.94
F5	C	30	382.21±3.75	-0.47	2.30±1.24	30	407.17±3.07	-1.85	0.75±1.18
	LLC	31	384.85±4.14	-2.64 *	-0.72±1.19	31	414.27±2.32	-1.28	0.23±1.01
	HLC	32	398.99±5.04	-2.16 *	-0.04±1.22	31	413.14±3.50	0.27	-1.76±0.92
F7	C	32	359.93±3.41	-2.60 *	0.04±0.75	32	359.93±3.41	-2.60 *	0.32±0.99
	LLC	31	370.63±2.26	-2.21 *	-1.53±1.14	31	370.63±2.26	-2.21 *	1.18±0.92
	HLC	33	369.60±2.79	-0.28	-1.30±2.23	33	369.60±2.79	-0.28	2.43±1.15

LLC-Low lead concentration; HLC- High lead concentration;
R- right wing; L-left wing
$p<0.05$*, $p<0.01$**, $p<0.001$***

2.60, $p<0.05$), and the control and HLC (t=-2.21, $p<0.05$).

The FA statistics for the wing length and width of both sexes in the lead treated groups and the control in the F3, F5, and F7 generation is shown in Table 2.

The indices used here reveal the association between the canalization and FA across traits, and

Table2. The fluctuating asymmetry change of wing length and width in both sexes of D.subobscura, for seven generations with and without lead

FA4= var(R- L)									
LENGTH		males				females			
generation	treatment	N	*mean*	*variance*	*F test*	N	*mean*	*variance*	*F test*
	C	33	1.01	25.5	1.45	33	(-)1.1	54.68	1.34
F3	LLC	33	0.09	17.57	1.02	31	4.12	40.82	1.93
	HLC	33	1.12	26.11	1.49	33	1.42	28.37	1.44
	C	30	0.73	21.99	1.18	30	-0.43	31.19	1.104
F5	LLC	31	1.36	26.02	1.82	31	1.43	34.43	1.54
	HLC	32	3.05	40.05	1.54	31	-1.60	20.27	1.7
	C	32	-0.28	19.75	2.06 *	33	-0.67	28.45	1.03
F7	LLC	31	-3.21	40.76	7.92 ***	32	0.78	27.53	1.2
	HLC	33	-2.91	156.49	3.84 ***	34	-1.45	34.20	1.24
WIDTH									
	C	33	0.12	27.98	1.85	33	0.30	69.92	0.16
F3	LLC	33	1.75	51.74	1.03	31	1.10	41.95	2.35 **
	HLC	33	-0.02	28.79	1.8	33	-1.38	29.69	1.41
	C	30	2.3	46.15	1.05	30	0.75	42.41	1.32
F5	LLC	31	-0.72	44.10	1.03	31	0.23	32.15	1.61
	HLC	32	-0.04	47.71	1.08	31	-1.76	26.29	1.22
	C	32	0.04	18.18	2.20 *	33	0.32	32.88	1.2
F7	LLC	31	-1.53	40.01	9.06 ***	32	1.18	27.46	1.37
	HLC	33	-1.30	164.67	4.11 ***	34	2.43	45.01	1.64

LLC-Low lead concentration; HLC- High lead concentration;

R –right wing; L-left wing

$p<0.05$*, $p<0.01$**, $p<0.001$***

has often been promoted as a general indicator of environmental stress at population and individual levels (Leung and Forbes, 1996; Møller and Swaddle, 1997). However, to validate the use of FA and DI as decision tools in conservation biology we need to know their accuracy and reliability as indicators of stress.

There are some literature data about the association between environmental stress and an increasing FA. Examples include the sharp increase in FA of the wing length and arista branching in *Drosophila* species in response to high temperature as an environmental stress factor (Imasheva et al. 1997). Moreover, developmental instability increases with different kinds of environmental factors, such as exposure to chemical pollutants (Valentine and Soule, 1973).

The present results of the variability of fluctuating asymmetry for the wing length and width during 7 generations of lead treatment in laboratory conditions in both sexes showed that the FA4 index (Palmer, 1994) is a more sensitive indicator than FA1. FA4 index analysis showed that the flies reared on the higher lead concentration (HLC) were in developmental homeostasis during several generations of selection pressure in laboratory conditions, except for the males of the F7 generation on HLC which exhibited increased FA. A possible explanation is inbreeding caused by strong environmental selection pressure and its influence on developmental homeostasis of males reared on the higher concentration of lead. There is also variability in FA and a significant difference between higher lead concentration and the other two experimental groups. Our results showed consistency with the published literature results that fluctuating asymmetry can increase when the strength of environmental stress exceeds a certain critical level (Clarke and McKenzie, 1992). They confirmed different, sex-related responses to this kind of environmental stress during several generations under lead in laboratory conditions. It follows that developmental stability can be a sensitive indicator of the physiological state of individuals in laboratory populations after several generations of treatment.

The results presented in this paper differ from those presented by Stamenkovic-Radak et al. (2008) who showed that within both lead concentrations females had significantly higher FA indices for the wing width than males. A significantly higher FA was found only in females for the wing width FA between the control and the lower concentration of lead. The present and former studies differ in the natural populations brought up in laboratory conditions on lead, meaning that the difference in the results can be due to the population-specific response on lead pollution. Operating at the population level, a stressor may eliminate unstable phenotypes from the population (Polak et al., 2002).

The population response to heavy metal pollution depends on the genetic structure of the population and the evolutionary history of a particular population. Also, the pre-adaptations have the potential to reduce levels of FA in populations originating from stressed environments. As a population adapts to toxic conditions, the effect of stress is less evident in fitness traits (fitness of the population is increased). Consequently, a population becomes increasingly adapted to stress so the use of FA as an indicator of environmental stress is undermined when comparing natural populations with different evolutionary histories.

There is growing evidence from experimental and theoretical studies that fluctuating asymmetry is not a consistent index of stress (Leung and Forbes 1996; Bjorksten et al. 2000). It has a potential as an indicator of stress and is inexpensive and easily measured, but for statistical patterns of asymmetry variations to become a useful tool in conservation biology it is necessary to understand the genetic basis of the underlying developmental mechanisms. Our results also call for caution in the use of FA in biomonitoring and in attributing patterns of FA variation to particular forms of stress in natural populations.

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УТИЦАЈ ОЛОВА НА РАЗВОЈНУ СТАБИЛНОСТ КОД *DROSOPHILA SUBOBSCURA* ТОКОМ СЕЛЕКЦИЈЕ У ЛАБОРАТОРИЈСКИМ УСЛОВИМА

ЗОРАНА КУРБАЛИЈА[1], Б. КЕНИГ[1], ЈОВАНА ПЛАВША[3],
МАРИНА СТАМЕНКОВИЋ-РАДАК[1,2] и М. АНЂЕЛКОВИЋ [1,2]

[1]*Институт за биолошка истраживања "Синиша Станковић",* 11000 Београд, Србија
[2]*Биолошки факултет, Универзитет у Београду,* 11000 Београд, Србија
[3] *Природно-математички факултет,* 21000 Нови Сад, Србија

Флуктуирајућа асиметрија представља повећани степен варирања билатерално симетричних карактера у узорку индивидуа, и може да буде индикатор ремећења нивоа развојне стабилности под утицајем геномског и/или срединског стреса. Степен развојне нестабилности је анализиран код популације *Drosophila subobscura,* узгајане у лабораторијским условима током седам генерација на две различите концентрације олова. Резултати FA4 индекса су показали да генотипови који су узгајани на вишој концентрацији олова су у развојној хомеостази, осим мужјака у F7 генерацији, за оба посматрана параметра крила. Резултати ове студије указују на то да различит степен загађења оловом у лабораторијским условима може изазивати различите одговоре на селекцију код третираних популација.