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LIFE-HISTORY VARIATION OF *Drosophila subobscura* UNDER LEAD POLLUTION DEPENDS ON POPULATION HISTORY

Bojan KENIG[1], Aleksandra PATENKOVIĆ[1], Marko ANĐELKOVIĆ[1,2,3], Marina STAMENKOVIĆ-RADAK[1, 2]

[1]Institute for Biological Research "Siniša Stanković", Department of Genetics of Populations and Ecogenotoxicology, University of Belgrade, Belgrade, Serbia
[2]Faculty of Biology, University of Belgrade, Belgrade, Serbia
[3]Serbian Academy of Sciences and Arts, Belgrade, Serbia

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Contamination represents environmental stress that can affect genetic variability of populations, thus influencing the evolutionary processes. In this study, we evaluate the relationship between heavy metal contamination (Pb) and phenotypic variation, assessed by coefficients of variation (CV) of life-history traits. To investigate the consequences of population origin on variation of life history traits in *Drosophila subobscura* in response to different laboratory conditions we compared populations from relatively polluted and unpolluted environments. Prior to experiment, flies from natural populations were reared for two generations in standard *Drosophila* laboratory conditions. Afterwards, all flies were cultured on three different media: one medium without lead as the control, and the other two with different concentrations of lead. Coefficients of variation (CV) of life-history traits (fecundity, egg-to-adult viability and developmental time) were analyzed on flies sampled in generations F2, F5 and F8 from these three groups. In later generations samples from both polluted and unpolluted environments showed the increased fecundity variation on media with lead. This increase is expressed more in population from unpolluted environment. On contrary, population from unpolluted environment had increased variation of developmental time in earlier, F2 generation, compared to the population from polluted environment. Our results showed that the response to heavy metal contamination depends on the evolutionary history of the populations regarding habitat pollution.

Key words: Coefficient of variation (CV), *Drosophila subobscura*, Lead pollution, Life-history traits

Corresponding author: Bojan Kenig, Institute for Biological Research "Siniša Stanković", Department of Genetics of Populations and Ecogenotoxicology, University of Belgrade, Bul. despota Stefana 142, 11060 Belgrade, Serbia,Phone: +381 11 20 78 328; Fax: +381 11 27 61 433; E-mail: bojan.kenig@ibiss.bg.ac.rs

INTRODUCTION

Human activities contribute to environmental change in a great extent and the exposure of organisms to man-made pollution represents potential environmental stress as well as a novel source of selection pressure (VITOUSEK *et al*., 1997; HOFFMANN and MERILA, 1999; VAN STRAALEN and TIMMERMANS, 2002; JARUP, 2003). Organisms living in polluted environments may become tolerant to the stress factor they are exposed to. Increased tolerance to pollutants in population may be acquired at the individual level through acclimation, through phenotypic plasticity, or through natural selection acting on individuals (KLERKS and WEIS, 1987; HOFFMANN and PARSONS, 1994; LOPES *et al*., 2004; MEDINA *et al*., 2007; LAGISZ and LASKOWSKI, 2008). One aspect of the adaptive response includes modification of life-history traits (MALTBY *et al*., 1987; HOFFMANN and PARSONS, 1994; FORBES and CALOW, 1997; SHIRLEY and SIBLY, 1999; AGRA *et al*., 2011; FISKER *et al*., 2011; SARO *et al*., 2012). It has been shown that lead affects fecundity, viability and developmental time in *Drosophila subobscura*, with generations of exposure and lead concentration as factors (STAMENKOVIC-RADAK *et al*., 2008; KENIG *et al*., 2013). The study on the same species indicated that the presence of lead in higher concentration over extended period of time may reduce the stability of wing morphology and consequently reduce the fitness of exposed individuals (KURBALIJA NOVIČIĆ *et al*, 2012).

Evolutionary alteration of life-history pattern in response to pollutants depends on the presence of genetic variability for life history traits (FALCONER, 1989). Numerous field studies showed that history of metal pollution imposed selection on exposed populations (KLERKS and WEIS, 1987; POSTHUMA and VAN STRAALEN, 1993), and heavy metals as specific type of environmental stress seem to affect genetic variability (HOFFMANN and HERCUS, 2000; LOPES *et al*., 2006; RIBEIRO *et al*., 2012). Nevertheless, it is still not clear whether this type of environmental stress leads to the increased or decreased genetic variation (BOURRET *et al*., 2008). Increased levels of heavy metal contamination are usually associated with reduced genetic variation at the population level, which can limit the ability of adaptive response to environmental stress (KIM *et al.,* 2003; BOURRET *et al*., 2008; UNGHERESE *et al*., 2010; RIBEIRO *et al*., 2012). On the other hand, stressful conditions that are rarely encountered by populations can also lead to increased genetic variation (HOFFMANN and MERILA, 1999; HOFFMANN and HERCUS, 2000; VAN STRAALEN and TIMMERMANNS, 2002).

Differences in resistance to heavy metal pollution between populations from different environments depend on their evolutionary history (KLERKS and LEVINTON, 1989), and these differences are often interpreted as local adaptations (REZNICK and GHALAMBOR, 2001; KAWECKI and EBERT, 2004). A common approach to examine the presence of genetic differences in resistance to pollution involves rearing individuals originating from differently polluted environments in common, usually optimal laboratory conditions in order to eliminate most of the environmental variation as well as variation resulting from genotype by environment interactions (KLERKS *et al*., 2011). However, one cannot fully exclude the possibility that such environments differ in characteristics other than pollution level. An alternative approach, allowing the manipulation of a single variable, is to study the potential for the evolution of resistance to contaminants using some of laboratory selection protocols. Therefore, we used both approaches in the current study by subjecting populations originating from the environments with different levels of heavy metal pollution to selection for the increased resistance to lead in laboratory conditions (XIE and KLERKS, 2003). It can be assumed that life history traits are under either stabilizing or directional selection in the presence of environmental stress. Our goal was to find out whether lead pollution leads to increased or decreased levels of quantitative genetic variation of life-history traits, and to measure the intensity of stabilizing selection in these populations using coefficients of variation (CV).

MATERIALS AND METHODS

We sampled two populations of *Drosophila subobscura* in Serbia (Deliblato Sands-DS and Botanical Garden-BG) by using fermented fruit traps. Deliblato Sands (DS) (*Orno-Quercetum cerris-virgiliane*) is a locality characterized by the lowest content of heavy metals in the soil on the territory of Serbia, with lead being at the lowest level compared to other heavy metals (16,39-35,00 mg/kg) (KADOVIĆ and KNEŽEVIĆ, 2002). It is located about 60 km north-east from Belgrade, Serbia (i.e. 44° 49' N; 21° 07' E). The second locality is Botanical Garden (BG) (Arboretum), located in the urban part of Belgrade (i.e. 44° 49' N; 20° 28' E), which has been chronically exposed to anthropogenic activity. The average concentration of lead in the soil of this area is 298.6 mg/kg (GRZETIC and GHARIANI, 2008; MARJANOVIĆ *et al*., 2009).

The flies were attracted and collected using fermented fruit traps. Approximately 100 isofemale lines (IF) per population were made, each from a single gravid wild-caught female. All lines were maintained and all experiments performed under constant laboratory conditions, at 19°C, approx. 60% relative humidity, light of 300 lux and 12/12 h light/dark cycles. After two generations, three pairs of males and females from each IF line were used to establish two synthetic, mass populations in order to preserve the original genetic variability. The F1 progeny from these mass populations was used to establish the experimental groups on three different media. The control group was reared on standard *Drosophila* medium (water/corn-meal/yeast/sugar/agar/nipagine), without any lead. The first experimental group (the low lead concentration group – LLC) had 10 µg/mL of lead acetate added to the medium [Pb $(CH_3COO)_2 \cdot 3H_2O$], which is a sublethal concentration. The second experimental group (the high lead concentration group – HLC) had 100 µg/mL of lead acetate added which represents the lethal concentration of 20% (LC 20). All groups were kept *en masse*, 10 bottles each. The flies from the F1 generation were mixed randomly within each group and used as parents of F2. Each subsequent generation (8 in total) was obtained by mixing the parents collected from different bottles within the group.

In order to examine whether the acquisition of tolerance to lead involved changes in life-history patterns, we recorded fecundity, egg-to-adult viability, and developmental time for each line from each population, in generations F2, F5, and F8. Twenty-five virgin females (three days old) were chosen arbitrarily from the previous generation of mass cultures from each experimental group. Females were used as parental generation in further experiment and were placed individually in vials with standard, LLC and HLC medium to mate with two males (three days old) from the same IF line. After laying eggs the females were transferred to new vials every 24 hours. The number of eggs laid was recorded during six consecutive days, and the data from the fourth, fifth, and sixth day were taken as a measure of the early, three-day fecundity. All vials were kept and checked for number of the enclosed adults. The developmental time (DT) was recorded, once all the adults had emerged, by the formula: $DT = \sum nd \cdot d / \sum nd$, where *nd* is the number of flies emerging and *d* represents the days after the eggs were laid. The egg-to-adult viability was calculated as the ratio of the emerged adults to the number of laid eggs, expressed as: $V = n/N$, where *N* is the number of eggs and *n* is the number of adults emerging from the *N* eggs. The number of observations for all the traits per experimental group was equal to the number of vials with females per group.

The statistical analysis was based on the number of observations per experimental group, for each trait - fecundity, egg-to-adult viability, and developmental time. Viability data needed transformation and were analysed as arcsine-square-root transformed proportions. Normality and homogeneity of data variances were, in general, confirmed by Jarque-Bera, Bartlett's and Levene's tests. The assumption of normality of variances was met in almost all, and in a few cases the assumption of homoscedascity was violated, so Welch ANOVA was used. However, ANOVAs are quite resistant to deviations from homogeneity of variances as long as sample sizes are large (ZAR, 1999). Post-hoc Tukey HSD test was run within two-way ANOVA to resolve the variability between populations, generations and concentrations of lead for three fitness components (SOKAL and ROHLF, 1995). The sequential Bonferroni procedure (RICE, 1989) was used to test statistical significance of all comparisons. For comparison of coefficients of variation Fligner-Killeen test was used, which includes z statistics. All analyses were performed using Statistica for Windows 5.0 (StatSoft Inc., Aurora, CO, USA) and PAST software (HAMMER *et al*., 2001).

RESULTS

Intra- and inter-population differences in coefficients of variation of fecundity

Table 1. Coefficients of variation (CV) of fecundity in control group (C), group reared on lower lead concentration (LLC) and group reared on higher lead concentration (HLC) originating from Deliblato Sands (DS) and Botanical Garden population (BG); Gen. – generation.

	control group		LLC group		HLC group	
Gen.	DS	BG	DS	BG	DS	BG
F2	15.48	30.79	22.46	20.02	16.15	18.50
F5	16.69	20.85	9.28	13.64	19.18	12.54
F8	24.73	27.66	40.71	35.36	45.10	28.88

In the group originating from Deliblato Sands, reared under lower lead concentration, CV of fecundity decreases in F5, when compared to F2 generation ($p < 0.001$). In F8 there is significant increase of this parameter in both lead treated groups (LLC and HLC) compared to both F2 and F5 generations (LLC F2/F5: $p < 0.001$; LLC F5/F8: $p < 0.004$; LLC F2/F8: $p < 0.027$; HLC F2/F8: $p<0.001$; HLC F5/F8: $p < 0.001$).

A significant increase of CV of fecundity in LLC group from Botanical Garden is evident in F8 generation, compared to other generations (F2/F8: $p < 0.012$; F5/F8: $p < 0.000$). In HLC group of the same population, there are significant differences in coefficients of variation between all generations; a decrease of CV in F5 (F2/F5: $p < 0.040$) followed by the increase in F8 (F2/F8: $p < 0.011$; F5/F8: $p < 0.001$).

The value of CV of fecundity is higher in the control group from BG in F2 generation ($p < 0.011$) compared to the control group from DS. In F5 generation, CV value of LLC group from BG sample is significantly higher compared to DS ($p < 0.042$) (Table 1).

Compared to the earlier generations, the coefficient of variation of fecundity in HLC groups is increased in generation F8, in both populations. This is more expressed in HLC group from DS compared to BG ($p < 0.021$).

Intra and inter-population differences in coefficients of variation of egg-to-adult viability

Table 2. Coefficients of variation (CV) of egg-to-adult viability in control group (C), group reared on lower lead concentration (LLC) and group reared on higher lead concentration (HLC) originating from Deliblato Sands (DS) and Botanical Garden population (BG); Gen. – generation.

	control group		LLC group		HLC group	
Gen.	DS	BG	DS	BG	DS	BG
F2	13.17	9.03	16.62	11.32	10.05	12.6
F5	9.73	12.98	14.69	10.80	12.41	9.34
F8	18.53	13.97	18.75	15.47	12.40	12.81

Generally, there is no significant difference in coefficients of variation for viability between groups (lead concentrations), generations and populations. A significant increase of CV for viability in F8 generation when compared to F5 ($p < 0.002$) is found only in the control group originating from DS, while in BG control group significant increase of CV is evident in F8 compared to F2 generation ($p < 0.014$). In F2 generation, viability CV is significantly higher in LLC group of DS than of BG ($p < 0.028$).

Intra- and inter-population differences in coefficients of variation of developmental time

Table 3. Coefficients of variation (CV) of developmental time in control group (C), group reared on lower lead concentration (LLC) and group reared on higher lead concentration (HLC) originating from Deliblato Sands (DS) and Botanical Garden population (BG); Gen. – generation.

	control group		LLC group		HLC group	
Gen.	DS	BG	DS	BG	DS	BG
F2	1.899	1.248	6.578	0.635	4.751	1.681
F5	2.382	1.762	2.737	1.321	2.637	1.121
F8	2.428	1.499	1.549	2.113	3.351	1.836

The variation of developmental time is the highest in groups of individuals originating from Deliblato Sands in F2 generation treated with lower lead concentration (Table 3), but it decreases in later generations (F2/F5: $p < 0.001$; F2/F8: $p < 0.001$; F5/F8: $p < 0.019$). The highest value of CV in HLC group is as well obtained in F2 generation. Significant differences for developmental time in this group are evident between F2 and F5 generations ($p < 0.013$).

Regarding the BG population, a significant increase of CV value for developmental time through generations is recorded in LLC group (F2/F5: $p < 0.005$; F2/F8: $p < 0.000$; F5/F8: $p < 0.037$). In generation F5, there is a significant decrease in CV for HLC group, which is evident by significant differences of the coefficient's value between F5 and the other generation (F2/F5: $p < 0.037$; F5/F8: $p < 0.039$).

The only significant difference of CV value between populations in the control group is evident within generation F8, where DS has higher CV compared to BG ($p < 0.022$). Group LLC of DS has significantly higher value of CV for developmental time when compared to corresponding group of BG in F2 ($p < 0.000$) and F5 generation ($p < 0.003$). The values of CV in HLC groups are different between DS and BG populations, in all the analyzed generations in the same pattern; it is higher in HLC group of DS compared to BG (F2: $p < 0.001$; F5: $p < 0.007$; F8: $p < 0.028$). As in the LLC group, CV has higher level of inter - generational variation in the sample from DS population.

Generally, the variation of developmental time is higher in groups originating from Deliblato Sands which is mostly expressed in F2 generation on lead polluted mediums.

DISSCUSSION

Heavy metals, as a type of environmental stress, can lead to changes in quantitative genetic variation in exposed populations. We aimed to quantify levels of life history traits variation under conditions of different lead pollution in two populations of *D. subobscura.* The significant inter-population differences of CV of fecundity in the control groups in F2 generation indicate the variability between natural populations. In F2 generation, DS has lower CV compared to BG, regardless of the lead treatment. In later generations CV increased in the groups treated with lead and originating from DS population, which is probably due to the expression of cryptic genetic variation in this population. The results are consistent with the notion that stressful conditions rarely encountered by populations can lead to increased rates of recombination and mutation that consequently leads to increased genetic, and therefore phenotypic, variation (HOFFMANN and MERILA, 1999; HOFFMANN and HERCUS, 2000). On the other hand, population BG shows little or no variation in CV of fecundity through generations, which indicates that BG population possessed lower level of genetic variability for this trait prior to the experiment. Considering that, it is showed that Botanical Garden population is preadapted to heavy metal pollution (KENIG *et al*, 2010; KURBALIJA NOVICIC *et al*, 2012) and lower variability of particular traits can be the consequence of selection pressure in natural habitat of this population and a cost (trade off) of increased tolerance to heavy metals.

Exposure of *D. subobscura* individuals to different concentrations of lead does not cause significant changes in variation of egg to adult viability. The same level of variation under lead pollution in both populations and through generations is probably preserved through mechanisms of resistance to heavy metal pollution. Viability is important fitness component and it is under stabilizing selection, canalized against environmental perturbations (STEARNS, KAISER and KAWECKI, 1995), which is confirmed by relatively low coefficients of variation of this trait in all groups and by absence of differences between the experimental groups.

The coefficient of variation of developmental time shows population specific response. The population from a relatively unpolluted environment, Deliblato Sands, has increased variation of developmental time, compared to the Botanical Garden. Also, the variation of developmental time is higher in groups of individuals treated with both lead concentrations originating from

Deliblato Sands in F2 generation. Again, this indicates that the lead pollution represents a stress for individuals originating from unpolluted environment reflected in lower phenotypic variability. Individuals from the Botanical Gardens showed lower level of variability for developmental time both in the control and lead conditions in almost all generations examined compared to the Delibato Sands population. It is possible that Botanical Garden population passed through a directional selection and hence suffered genetic erosion due to historical exposure to pollution. Directional shift in the survival curve occurred towards increase in survival under both lead contaminated environments. At the same time, the level of overall genetic variability of population was lowered by exclusion of sensitive genotypes and led to interpopulation differences in trait variation under laboratory conditions (SWINDEL and BOUZAT, 2006). Nevertheless, it is evident that directional selection in original population from Botanical Gardens did not reduce genetic variability for developmental time to a minimum determined by some environmental constraints. Similar type of acquiring resistance to contamination was found in studies of the effect of lead on fitness components in *D. subobscura* (STAMENKOVIC-RADAK *et al*., 2008, KENIG et al., 2012) as well as in studies with other organisms and pollutants (KLERKS and WEIS, 1987; REZNICK and GHALAMBOR, 2001; KLERKS, 2002). The diminishing of interpopulation difference through generations under laboratory conditions is mostly caused by a strong decrease of variation of this trait in experimental groups originating from Deliblato Sands. Higher level of variability of developmental time in Deliblato Sands, especially in the first generations of experiment, points to expression of higher level of genetic variability in this population, at least when it comes to genes included in determination of this complex trait. The stressful environment that is out of range that DS population encounters broke genetic buffer mechanisms and increased variation of time needed for development from egg to adult, as showed for other traits (RUTHERFORD, 2000, 2003). Taking into account that coefficients of variation are inverse measure of canalization and strength of stabilizing selection, our results indicate that the developmental time is under strong stabilizing selection. The obtained results for developmental time are in concordance with the view that all traits are involved in tradeoffs which must be taken into account when evaluating the sensitivity of fitness to changes in a trait. For example, in *Drosophila* an increase in fecundity usually involves an increase in weight at eclosion that can only be achieved by prolonging development. This means that neither fecundity nor development time can be under directional selection, for to change one in a direction that increases fitness implies a change in the other that decreases fitness (STEARNS, KAISER and KAWECKI, 1995, ZWANN *et al*., 1995).

Increased levels of genetic variation are often found in the environments characterized by extreme abiotic conditions (HOFFMANN and MERILA, 1999; HOFFMANN and HERCUS, 2000). Higher level of genetic variation is probably the result of expression of previously neutral genes that were not eliminated by natural selection (GUNTRIP *et al*., 1997), i.e. expression of cryptic genetic variability (MASEL, 2006; MCGUIGAN and SGRO, 2009). Important aspect of this perspective is that most of variants of traits induced by stress would be quickly eliminated by natural selection in new environment because it would represent deleterious phenotypes (RUTHERFORD and LINDQUIST, 1998; QUEITCSCH, SANGSTER and LINDQUIST, 2002). It is not clear, however, whether the increase in genetic variation is because these conditions are stressful, or because they are novel or rarely encountered. HOLLOWAY *et al*., (1990) and GUNTRIP *et al*., (1997) tested prediction of increase of genetic variability due to expression of genes that were inactive in native environment. In both cases, the results confirmed the prediction. These and similar studies rely on a quantitative genetic assumption that contribution of each gene in overall phenotypic variability is small and equal

(FALCONER, 1989). Level of genetic variability could increase quickly after the transfer of population into the novel environment, which suggests that the expression of the genes that are already present is realistic possibility (HOLLOWAY *et al*., 1990).

We showed that fitness components in *D. subobscura* are differentially canalized with respect to environmental stress and that developmental time is under strong stabilizing selection. The analyzed fitness traits show different levels of variability in response to treatment with lead, and it is expected that differences in variation of developmental time can lead to changes in fitness of different genotypes over time.

This study confirmed that the response of populations from differently polluted environments to lead contamination depends on their evolutionary history, that changes in genetic variability can be translated into phenotypic variability after transfer into the stressful (novel) environment, which can, subsequently, be used for ecotoxicological studies as a long-term indication of environmental pollution.

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VARIRANJE OSOBINA ŽIVOTNE ISTORIJE KOD *Drosophila subobscura* U USLOVIMA ZAGAĐENJA OLOVOM ZAVISI OD POPULACIONE ISTORIJE

Bojan KENIG[1], Aleksandra PATENKOVIĆ[1], Marko ANĐELKOVIĆ [1,2,3], Marina STAMENKOVIĆ-RADAK [1,2]

[1]Institut za biološka istraživanja "Siniša Stanković", Odeljenje za genetiku populacija i ekogenotoksikologiju, Univerzitet u Beogradu, Beograd, Srbija
[2] Biološki fakultet, Universitet u Beogradu, Beograd, Srbija
[3] Srpska akademija nauka i umetnosti, Beograd, Srbija

Izvod

Zagađenje teškim metalima, kao sredinski stres, može dovesti do promena u osobinama životne istorije organizama i na taj način do promene u nivou kvantitativno genetičke varijabilnosti u populacijama. Kako bi ispitali uticaj populacione istorije zagađenja teškim metalima na variranje osobina životne istorije kod *Drosophila subobscura*, poredili smo populacije ove vrste poreklom iz relativno zagađene i nezagađene sredine. Po donošenju jedinki ove vrste iz prirode one su gajene na standardnom Drosophila supstratu tokom dve generacije, posle čega su postavljane na tri različita supstrata u laboratorijskim uslovima: supstratu bez olova – kontrolna grupa, i dva supstrata sa različitim koncentracijama olova. Koeficijenti varijacije za tri osobine životne istorije (fekunditet, vijabilitet jaje – adult, i brzina razvića) su analizirani u drugoj, petoj i osmoj generaciji takvog tretmana. Jedinke poreklom iz zagađene ali i nezagađene sredine su pokazale povećanu varijabilnost fekunditeta kada su gajene na supstratima sa olovom, ali samo u kasnijim generacijama. Ovo povećanje varijabilnosti je jače izraženo u populaciji iz nezagađene sredine. Takođe, postoji populaciono – specifičan odgovor jer populacija iz nezagađene sredine ima povećanu varijabilnost brzine razvića u generaciji F2 u odnosu na populaciju iz zagađene sredine. Rezultati ukazuju na to da odgovor na zagađenje teškim metalima na nivou genetičke varijabilnosti zavisi od evolucione istorije populacija.

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